mTOR REGULATES AURORA A VIA ENHANCING PROTEIN STABILITY

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Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Biochemistry and Molecular Biology,
Indiana University

December 2013

Accepted by the Graduate Faculty, of Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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DEDICATION

I dedicate this thesis to my family: to my parents, Xiu Zhu Fan and Shu Qin Yang, who have been loving, supporting, and encouraging me from the beginning of my life; to my husband Fei Huang, who provided unconditional support and encouragement through these years; to my son, David Yan Huang, who has made my life highly enjoyable and meaningful.

ACKNOWLEDGMENTS

I sincerely thank my mentor Dr. Lawrence Quilliam for his guidance, motivation, support, and encouragement during my dissertation work. His passion for science and the scientific and organizational skills I have learned from Dr. Quilliam made it possible for me to achieve this accomplishment.

Many thanks to Drs. Ron Wek, Mark Goebl, Maureen Harrington, and Simon Atkinson for serving on my committee and providing constructive suggestions and technical advice during my Ph.D. program.

I have had a pleasurable experience working with all the people in our laboratory. Thanks Drs. Justin Babcock and Sirisha Asuri, and Mr. Jeremiah Hendricks for providing reagents and technical assistance.

It was my great pleasure to work in a nurturing environment of the Department of Biochemistry and Molecular Biology at Indiana University, School of Medicine.

I would like to thank Ms. Lynn Jettpace, at the writing center in Indiana University and Purdue University at Indianapolis, for her consultations on my writings.

I am grateful to my employer Eli Lilly and Company for their sponsorship and strong support.

I would also like to thank my parents, my son, and all my family and friends. I would like especially to thank my husband Fei, who has been a continual source of support and encouragement from the beginning to the end of my graduate study.

Li Fan

mTOR REGULATES AURORA A VIA ENHANCING PROTEIN STABILITY

Mammalian target of rapamycin (mTOR) is a key regulator of protein synthesis. Dysregulation of mTOR signaling occurs in many human cancers and its inhibition causes arrest at the G1 cell cycle stage. However, mTOR's impact on mitosis (M-phase) is less clear. Here, suppressing mTOR activity impacted the G2-M transition and reduced levels of M-phase kinase, Aurora A. mTOR inhibitors did not affect Aurora A mRNA levels. However, translational reporter constructs showed that mRNA containing a short, simple 5'-untranslated region (UTR), rather than a complex structure, is more responsive to mTOR inhibition. mTOR inhibitors decreased Aurora A protein amount whereas blocking proteasomal degradation rescues this phenomenon, revealing that mTOR affects Aurora A protein stability. Inhibition of protein phosphatase, PP2A, a known mTOR substrate and Aurora A partner, restored mTOR-mediated Aurora A abundance. Finally, a non-phosphorylatable Aurora A mutant was more sensitive to destruction in the presence of mTOR inhibitor. These data strongly support the notion that mTOR controls Aurora A destruction by inactivating PP2A and elevating the phosphorylation level of Ser51 in the "activation-box" of Aurora A, which dictates its sensitivity to proteasomal degradation. In summary, this study

is the first to demonstrate that mTOR signaling regulates Aurora-A protein expression and stability and provides a better understanding of how mTOR regulates mitotic kinase expression and coordinates cell cycle progression. The involvement of mTOR signaling in the regulation of cell migration by its upstream activator, Rheb, was also examined. Knockdown of Rheb was found to promote F-actin reorganization and was associated with Rac1 activation and increased migration of glioma cells. Suppression of Rheb promoted platelet-derived growth factor receptor (PDGFR) expression. Pharmacological inhibition of PDGFR blocked these events. Therefore, Rheb appears to suppress tumor cell migration by inhibiting expression of growth factor receptors that in turn drive Rac1-mediate actin polymerization.

Lawrence A. Quilliam, Ph.D., Chair

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LIST OF ABBREVIATIONS

4E-BP1	Eukaryotic initiation factor 4E-binding protein 1
5’ TOP	5′ terminal oligopyrimidine tract
AMPK	Adenosine monophosphate-activated protein kinase
APC	Anaphase-promoting complex
ATM	Ataxia telangiectasia mutated
ATP	Adenosine-5'-triphosphate
AURKA	Aurora kinase A
Cdc20	Cell-division cycle protein 20
Cdc42	Cell-division cycle protein 42
Cdh1	Cdc20 homologue 1
CDK1	Cyclin dependent kinase1
DAPI	Diamidino-2-phenylindole
DEPTOR	DEP domain-containing mTOR-interacting protein
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
eIF4A	Eukaryotic Initiation Factor 4A
eIF4B	Eukaryotic Initiation Factor 4B
eIF4E	Eukaryotic Initiation Factor 4E
eIF4F	Eukaryotic Initiation Factor 4F
eIF4G	Eukaryotic Initiation Factor 4G
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FKBP12	FK506 binding protein 12
Frz1	Fizzy-related 1
G1	Gap 1
G2	Gap2
GAP	GTPase activating protein
GAPDH	Glyceraldehyde-3-P dehydrogenase
GDI	GDP-dissociation inhibitory factor
GDP	Guanine 5'-Diphosphate
GF	Growth factor
GST	Guanosine S-transferase Fusion Protein
GTP	Guanosine-5'-triphosphate
IGF	Insulin-like growth factor
IKKβ	IκB kinase β

M phase	Mitosis phase
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
mLST8	Mammalian lethal with Sec13 protein 8
mRNA	Messenger ribonucleic acid
mSIN1	Mammalian SAPK interacting protein
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
NCBI	National Center for Biotechnology Information
NFκB	Nuclear factor kappa-activated B cells
OA	Okadaic acid
PAGE	Polyacrylamide gel electrophoresis
PAK	P21-activated kinase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDK1	Phosphoinositide-dependent kinase-1
PI3K	Phosphatidylinositol 3-kinase
PI3P	Phosphatidyl inositol 3,4,5-trisphosphate
PKB	Protein kinase B
PLD	Phospholipase D
PMSF	p-methylsulfonylfluoride
PP2A	Protein phosphatase 2A
PP6	Protein phosphatase 6
PRAS40	Proline-rich Akt/PKB substrate 40 kDa
PROTOR-1	Protein observed with Rictor-1
PROTOR-1	Protein observed with Rictor-1
PTEN	Phosphatase and tensin homolog
Rac1	Ras-related C3 botulinum toxic substrate 1
RACE	Rapid Amplification of cDNA Ends
Raptor	Regulatory-associated protein of mTOR
Rheb	Ras homolog enriched in brain
Rho	Ras homologous
RICTOR	Rapamycin-insensitive companion of mTOR
S6K1	Ribosomal protein S6 kinase 1
SDS	Sodium dodecylsulfate
shRNA	Small hairpin RNA
siRNA	Small interfering RNA

Torin1	mTOR inhibitor 1
TPX2	Targeting Protein for Xenopus kinesin-like protein 2
TSC	Tuberous sclerosis
TSC1/2	Tuberous sclerosis complex 1/2
uORF	upstream Open Reading Frame
UTR	Untranslated regions
VEGF	Vascular endothelial growth factor
WT	Wild type

CHAPTER 1. INTRODUCTION

1.1 mTOR and Cancer

The Serine/Threonine kinase mammalian target of rapamycin (mTOR) is located at a critical point of convergence for the regulation of cell growth, differentiation, migration and survival [1, 2]. Dysregulation of mTOR signaling occurs in a diverse array of human cancers, including prostate, breast, ovarian, and brain tumors, which severely threaten human life [3, 4].

mTOR is known as mechanistic target of rapamycin or FK506 binding protein 12-rapamycin associated protein 1 (FRAP1), encoded by the *FRAP1* gene [1, 2]. Rapamycin is a specific mTOR antagonist that can block mTORC1 downstream signaling. Rapamycin analogs such as CCI-779 and RAD001 have exhibited impressive growth inhibitory effects against a broad range of human cancers in preclinical studies [5]. A new generation of mTOR inhibitors, which compete with ATP for binding the catalytic site of the kinase, has demonstrated potent and selective inhibition to both mTORC1 and mTORC2 activities [6] [7].

1.2 mTOR in PI3K/PTEN signaling pathway

The typical pathway of mTOR activation in signal transduction is via the PI3K/mTOR pathway.

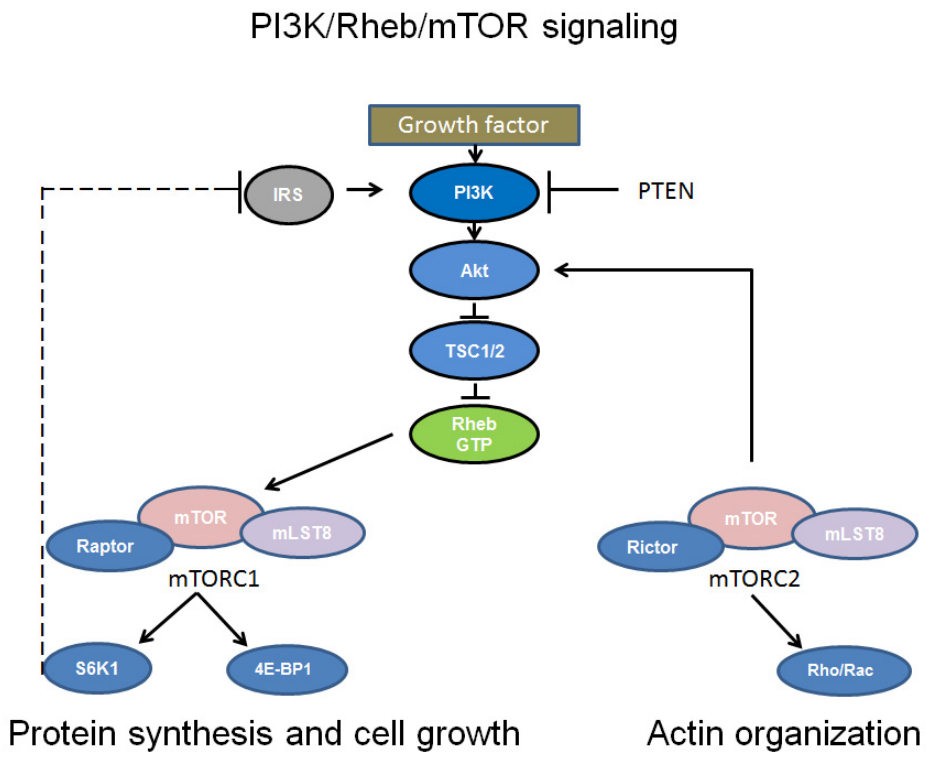

Figure 1.1 PI3K/mTOR pathway, see text for details.

Following stimulation with growth factors such as platelet derived growth factor (PDGF), cellular membrane receptor tyrosine kinases are phosphorylated and activated, resulting in recruitment of their substrates to the cell membrane, where they become tyrosine phosphorylated. Subsequently, recruitment and stimulation of phosphatidyl inositol 3 kinase (PI3K) activity produces the phosphatidylinositol second messenger phosphatidyl inositol 3,4,5-trisphosphate (PIP3). PIP3 binds to the serine/threonine kinase AKT and recruits it to the cell membrane, where it is activated through phosphorylation of Thr308 by phosphoinositide-dependent kinase 1(PDK1) plus a second protein kinase phosphoinositide-dependent kinase 1(PDK2) that phosphorylates Ser473. Activated AKT phosphorylates the tuberous sclerosis complex 1 and 2 (TSC1/2), relieving the small GTPase Rheb from the inhibitory GTPase activating protein GAP activity of TSC2. ERK activity downstream of Ras can also phosphorylate TSC2, inhibiting its Rheb-GAP activity. Rheb-GTP in turn activates the Ser/Thr kinase mTOR, which phosphorylates and activates ribosomal S6 kinase 1(S6K1) and eukaryotic translation initiation factor binding protein 1(4E-BP1). S6K1 regulates translation of TOP (5' terminal oligopyrimidine tract) mRNAs, which encode important components of the translation apparatus, while phosphorylation of 4E-BP1 promotes its dissociation from eukaryotic translation initiation factor 4E (eIF4E). The latter forms the initiation complex required for driving the 5’ cap-dependent translation of key cellular proteins including cyclin D1, Myc, and vascular endothelia growth factor. There is a negative feedback loop between S6K1 and insulin receptor substrate 1(IRS). S6K1-mediated

phosphorylation of IRS1 causes its destabilization, preventing the growth factor signaling to PI3K [8].

In addition to signals from growth factors that activate PI3K, it is anticipated that loss of the phosphatase and tensin homolog (PTEN) tumor suppressor, which is the phosphatase responsible for dephosphorylating the polyphosphoinositides generated by PI3K and is required for Akt activation, will also promote TSC2 down-regulation and subsequent activation of the Rheb/mTOR pathway.

1.3 mTORC1 vs. mTORC2

mTOR exists in two distinct complexes: mTOR complex 1(mTORC1) and mTOR complex 2 (mTORC2), which are different in their composition and substrate specificity [9]. mTOR complex 1 is composed of mTOR, a catalytic domain, mammalian lethal with Sec13 protein 8 (mLST8), proline-rich AKT substrate 40 kDa (PRAS40), DEP domain-containing mTOR-interacting protein (DEPTOR), and Raptor (regulatory-associated protein of mTOR)[9]. Raptor plays a role as a scaffold to present substrates to mTOR but does not alter kinase catalytic activity. PRAS40 is an inhibitory protein that blocks mTORC1 from binding substrates [11]. It is thought that Akt phosphorylation of PRAS40 at threonine 246 overcomes the inhibitory effect of PRAS40 on mTORC1 [12] [13]. The most characterized substrates of mTORC1 are initiation factor 4E-binding protein and S6 kinase 1. mTORC1 functions as a nutrient/energy/redox sensor and controls protein synthesis [3, 14, 15]. The activity of this complex is

stimulated by insulin, growth factors, serum, phosphatidic acid, amino acids (particularly leucine), and oxidative stress [16]. mTORC1 is sensitive to rapamycin, from the S6K1 point of view. In the second part (Chapter 4) of this thesis, I will address mTORC1 activities on gene expression and protein stability for a particular kinase, Aurora A, in human cancer cells.

mTOR complex 2 (mTORC2) contains mTOR, rapamycin-insensitive companion of mTOR (RICTOR), GβL, DEPTOR, and mammalian stress-activated protein kinase interacting protein 1 (mSIN1) [14, 17]. mTORC2 is rapamycin insensitive, except following prolonged exposure (>24 hours) to the drug [5]. mTORC2 phosphorylates Akt at ~~a~~ serine 473 (S473) located within ~~a~~ the C-terminal hydrophobic domain. Phosphorylation of this serine stimulates Akt phosphorylation at a threonine T308 residue by PDK1 and leads to full Akt activation. As such, mTORC2 appears to possess the activity of a previously elusive protein known as PDK2 [18] and as discussed in section 1.2. mTORC2 has also been shown to function as an important regulator of the cytoskeleton through its stimulation of F-actin stress fibers, paxillin, RhoA, Rac1, Cdc42, and protein kinase C α (PKCα) [19]. Regulation of mTORC2 is less well understood than that of mTORC1; however, mTORC2 activity seems to be stimulated by growth factors through the PI3 kinase pathway [11, 20-22].

There is crosstalk between mTORC1 and mTORC2. For example, cells treated with rapamycin for short time (1-2 h) inhibits mTORC1 activity, while longer treatment increases Akt phosphorylation, an mTORC2 indicator, via IRS1 degradation. Despite this breakthrough in the discovery of this feedback loop, the

complexity of this network is only just beginning to be understood. Recently, suppression of PI3 kinase was shown to decrease mTORC2 phosphorylation of Akt when cells were stimulated with growth factors insulin or insulin-like growth factors IGF in multiple cell lines [12, 13, 23], however details of the mechanisms still remain unclear. During the course of my thesis study, we encountered a similar question. That is, whether inhibiting mTORC1 activity would potentially increase mTORC2 function, in term of cell migration. In the first part (Chapter 3) of this thesis, I will address this issue.

1.4 Rheb as a molecular switch and upstream of mTORC1

Ras-homolog enriched in brain (Rheb) is a ~21 kDa small GTPase. Like other members of the Ras superfamily, the activity of Rheb is regulated by a GTP-GDP cycle. A guanine nucleotide exchange factor (GEF) is speculated to promote release of GDP from Rheb so GTP can bind. Rheb is active when GTP is bound, and this induces a conformational change that permits interaction with downstream effectors. But Rheb becomes inactive following hydrolysis of the bound GTP to GDP. GTPase activating protein (GAP) accelerates the rate of GTP hydrolysis of GTPase. The tuberous sclerosis complex, a tumor suppressor formed by the tuberous sclerosis complex 1 and 2 (TSC1/2) proteins, functions as a GAP that stimulates Rheb GTP hydrolytic activity and hence acts as a negative regulator of Rheb [24]. Translationally controlled tumor protein (TCTP) has recently been identified as a putative guanine nucleotide exchange factor for Rheb in Drosophila and humans [15, 25], but this has been questioned in mammalian cell [26]. The Proud lab re-evaluated the role of TCTP as a GEF for Rheb and did not find any evidence to support the model of TCTP being a GEF toward Rheb [27] [28]. It is likely that inhibition of Rheb GAP activity through ERK and Akt-mediated phosphorylation of TSC2 or IkB-mediated phosphorylation of TSC1, rather than the activation of a GEF, promotes Rheb-GTP accumulation. Rheb GAP can be activated by AMP activated protein kinase (AMPK) and this overrides the above inhibitory kinases to promote Rheb inactivation during nutrient deprivation [29-31].

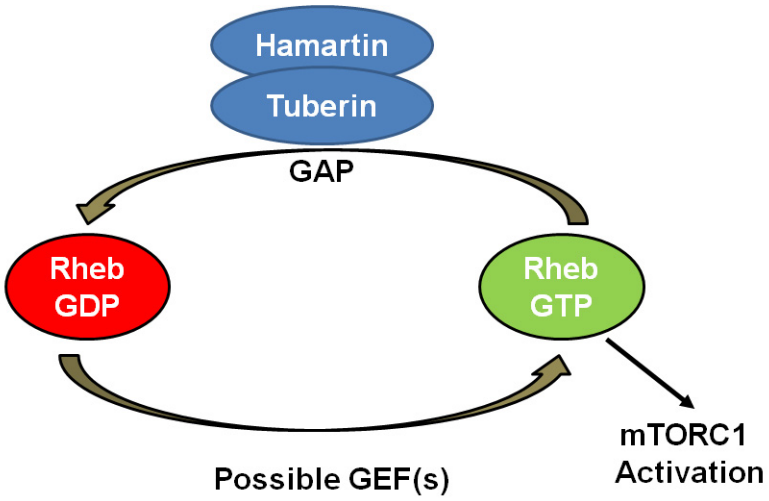

Figure 1.2 Rheb GDP-GTP cycle

The activity of Rheb is regulated by its guanine nucleotide binding states via a GTP-GDP cycle. GAP promotes GTP hydrolysis to GDP. TSC1/2 proteins functions as a GAP that stimulates GTP hydrolysis of Rheb, so Rheb is inactivated. A possible GEF releases GDP from Rheb so GTP can bind, and this induces a conformational change that permits interaction of Rheb with downstream effectors.

Genetic and biochemical studies have placed Rheb upstream of mTOR and down-stream of TSC1/2 in the PI3K/Akt/TOR/S6K signaling pathway [24] [26]. Both GDP- and GTP-bound Rheb directly bind to the TOR kinase domain but only GTP-bound Rheb activates the TOR catalytic function [17, 32].

A key structural feature of Rheb is its C-terminal CaaX box. C is the cysteine that is prenylated, a is any aliphatic amino acid, and the identity of X determines which enzyme acts on the protein. Farnesyltransferase recognizes CaaX boxes where X = M, S, Q, A, or C and catalyze Rheb activity. Farnesyltransferase inhibitor (FTI), originally designed to block Ras-induced cancer, can suppresses Rheb activity, and consequently inhibit mTOR activity and cell growth [24, 33-35].

Recent observations suggest that Rheb activates TOR by relieving an inhibitory interaction between TOR and FK506-binding protein 38 [18]. Very recently, it is thought that phospholipase D1(PLD1) also binds Rheb and can be activated by it [36, 37], but whether PLD1 also contributes to mTOR activation via generation of phosphatidic acid is not clear.

Additional evidence to support the notion that Rheb is upstream of mTORC1 is that overexpressed Rheb can restore the TOR-dependent phosphorylation of S6K following amino acid withdrawal. Rheb has been shown to bind to mTORC1 and its accessory protein raptor to stimulate mTOR kinase activity responsible for cell growth and cell cycle progression in some tumors. The mTOR specific inhibitor rapamycin can block Rheb-induced transforming activity [38].

An elegant work recently showed that specific activation of mTORC1 by Rheb G-protein *in vitro* involves enhanced recruitment of its substrate protein. Activation of mTORC1 by Rheb involves enhanced binding of 4E-BP1. Raptor plays an important role in the activation of mTORC1 by Rheb. Rheb does not induce autophosphorylation of mTOR [39, 40].

1.5 Rho family and cytoskeleton reorganization

Reorganization of the actin cytoskeleton is important in many cellular processes, including cell morphology change, cell adhesion and cell migration. Filamentous actin (F-actin) can be organized into four discrete structures - stress fibers, lamellipodia, membrane ruffles and filopodia. The Rho family, including ras homolog gene family member A (RhoA), ras-related C3 botulinum toxin substrate 1 (Rac1), and cell division control protein 42 homolog (Cdc42), control the actin cytoskeleton organization [41, 42]. RhoA mediates lysophophatidic acid -induced contractile stress fiber formation [43]. Rac1 is essential for growth factor-induced lamellipodia. Lamellipodia are thin protrusive actin sheets that dominate the edges of cultured cells, and membrane ruffles at the leading edge of cells are the result of lamellipodia that lift off the substratum and fold backward [44], while Cdc42 mediates bradykinin-promoted filopodia [42].

1.6 mTORC2 in Rho/Rac1-induced cell motility

To date, little is known about the role of Rheb in regulating cell migration. However, Rac1 activates F-actin polymerization at the leading edge of cells, assembling fans, termed lamellipodia, allowing directed movement [37]. In the presence of growth factors, chemotaxis plays a central role in various biological processes, such as metastasis of cancer cells. Because only activated, GTP-bound Rac can bind p21-activated kinase (PAK), use of PAK to pull down Rac-GTP often is an approach to characterize active Rac1, and it can be used as a read-out for Rac1 activation. It is well established that Rac1 is activated downstream of receptor tyrosine kinases via multiple GEFs (independently of mTORC1) and Rac1 activation promotes actin polymerization [45-47]. Recent studies also reported that mTORC2 seems to function upstream of Rho GTPases to regulate the actin cytoskeleton.

It was reported that mTORC2 may function as an upstream of Rho GTPases to regulate the actin cytoskeleton [46]. In mTOR, mLST8 or rictor siRNA-transfected cells, expression of constitutively active form of Rac1 (Rac1-L61) or RhoA (RhoA-L63) restored organization of the actin cytoskeleton, indicating that mTORC2 may regulate the actin cytoskeleton through RhoA and Rac1. mTORC2 mediated, serum/ insulin induced actin polymerization is rapamycin insensitive in NIH 3T3 cells [46]

Additionally, mTORC1 represses mTORC2 through feedback mechanisms, including inhibition of insulin signaling and platelet derived growth factor receptor (PDGFR) expression [31, 48]. Rapamycin treatment has been

shown to relieve this inhibition leading to mTORC2 activation in multiple cancer cell lines [23]. Recent data have implicated the TORC2 kinase as the major hydrophobic kinase that phosphorylates Ser473 on Akt [49]. Elevated Akt kinase activity is found in a majority of glioblastomas (GBM) [50], in which mTORC2 hyperactivated and promoted tumor cell proliferation and invasive potential. In contrast, shRNA-mediated inhibition of rictor expression reduces TORC2 activity, cell growth, and migration [49]. Whether Rheb is involved in these pathways to mediate Rac activation and cell motility in GBM cells will be addressed in chapter 4.

1.7 mTORC1 signal and its substrates S6K1 and 4EBP1

The best-characterized substrates of mTORC1 are 4E-BP 1and S6K1. mTORC1 phosphorylates S6K1 on a threonine residue (T389) [19][20]. This event stimulates the subsequent phosphorylation of S6K1 by PDK1 [20][21]. Active S6K1 in turn stimulate the initiation of protein synthesis through activation of S6 ribosomal protein (a component of the ribosome) and other components of the translational machinery [22].

In most cases, the rate of translation is regulated at the initiation phase, when a ribosome is recruited to the 5'end of an mRNA. The 4E-BP1 inhibits translation initiation by binding to the translation factor eIF4E, and prevent recruitment of the translation machinery to mRNA. The 4E-BP1 inhibits translation in a reversible manner. Hypophosphorylated 4E-BP1 interacts avidly with eIF4E,under stimulation of cells with hormones, cytokines, or growth factors, results in activating mTORC1, whereas hyperphosphorylated 4E-BP1 consequently abrogates its eIF4E-binding activity, resulting in inactivating mTORC1.

mTORC1 has been shown to phosphorylate at least four residues of 4E-BP1, Threonine 37/46 (T37/46), Serine 65 (S65), and Threonine 70 (T70), in a hierarchical manner [51]. Phosphorylation on T37 and T46 are priming sites, followed by T70 phosphorylation and S65 last. However, phosphorylation of S65 and T70 sites alone is not sufficient to disrupt 4E-BP1 binding to eIF4E. Therefore, I chose 4E-BP1 T37/46 phosphorylation status as markers for evaluation of mTORC1 activity in this study.

Besides controlling 4E-BP1 phosphorylation, mTOR also regulates protein synthesis by a distinct mechanism: mTOR phosphorylates and activates the ribosomal protein S6 kinase, which phosphorylates S6 ribosomal protein, a component of the S40 ribosome subunit, thus facilitating protein translation. Activated S6K initiates the translation of a class of mRNAs containing a tract of polypyrimidine (TOP) in their 5'untranslated regions (UTR) [52].

1.8 mTOR inhibitors on S6K1 vs. 4EBP1

Rapamycin was the first drug found to inhibit mTOR activity. Indeed, its anti-fungal and immunosuppressant actions were known prior to the subsequent identification of yeast TOR and its mammalian counterpart [53-56]. Rapamycin first forms a complex with the intracellular receptor FK506 binding protein 12 (FKBP12) and then binds a domain distinct from the catalytic site of mTOR, blocking mTOR function. Rapamycin is highly specific for mTORC1, and its analogs are effective against certain types of cancer. These limited clinical successes led to researchers investigating the mechanisms of rapamycin action behind this failing. Firstly, rapamycin inhibits mTORC1, but not completely, which was demonstrated by rapamycin blocking S6K1, but not 4E-BP1 phosphorylation [55] [57]. My unpublished data supported this notion. Secondly, mTORC1 inhibition often results in feedback activation of mTORC2 as well as other upstream growth and survival signals [58]. Third, rapamycin does not directly inhibit the mTORC2 complex whose activity is required for the growth of several types of cancer. Recent intensive efforts have focused on developing a new generation of mTOR inhibitors that includes Torin1 [59, 60], KU63794, and PP242 [6, 61]. These inhibitors inhibit both mTORC1 and mTORC2 activities. Compared with rapamycin, these ATP competitive inhibitors impaired the proliferation of primary cells to a far greater degree [6, 7]. At the beginning of their development, it was thought that the ability of PP242 and Torin1 to block cell proliferation more efficiently than rapamycin could be a result of inhibition of mTORC2 in addition to mTORC1. However, in MEFs genetically deficient for

mTORC2 activity, rapamycin was also less effective at blocking cell proliferation than PP242 and Torin1. The data suggest that the potent inhibitory effect of PP242 and Torin1 on cell proliferation is a result of more complete mTORC1 inhibition, but not a consequence of both mTORC1 and mTORC2 inhibition [60]. Consistently, both PP242 and Torin1 had much greater effects than rapamycin on 4E-BP1 phosphorylation and cap-dependent mRNA translation. These molecules competed with ATP in the catalytic site of mTOR, by demonstrating strong inhibition of both S6K1 and 4E-BP1 (mTORC1) and mTORC2 as well, showing high efficacy in the preclinical setting [6, 7]. Based on these drugs' discovery during the period time of my research, I switched from rapamycin to using the ATP competitive inhibitors to study mTOR function in this thesis.

1.9 mTOR in transcriptional regulation

TOR signaling is a prerequisite for the induction of ribosomal protein (r-protein) gene transcription that occurs in response to improved nutrient conditions. This included the transcription of ribosomal RNA (rRNA) genes by RNA polymerase I (Pol I), transcription of ribosomal protein genes by RNA polymerase II (Pol II), and transcription of transfer RNA (tRNA) and 5S genes by RNA polymerase III [62, 63]. Recently, several studies identified a mammalian Pol I-specific transcription factor, transcription intermediary factor 1-alpha (TIF1A), whose activity is modulated by rapamycin. Mayer et al. [64] demonstrated that TIF-IA, an essential RNA Pol II transcription factor [63] is sufficient to rescue rapamycin-mediated inhibition of ribosomal DNA

transcription, demonstrating that mTOR plays a role in gene transcription. However, whether mTOR regulates Aurora A transcription is unknown.

1.10 mTOR in translational regulation

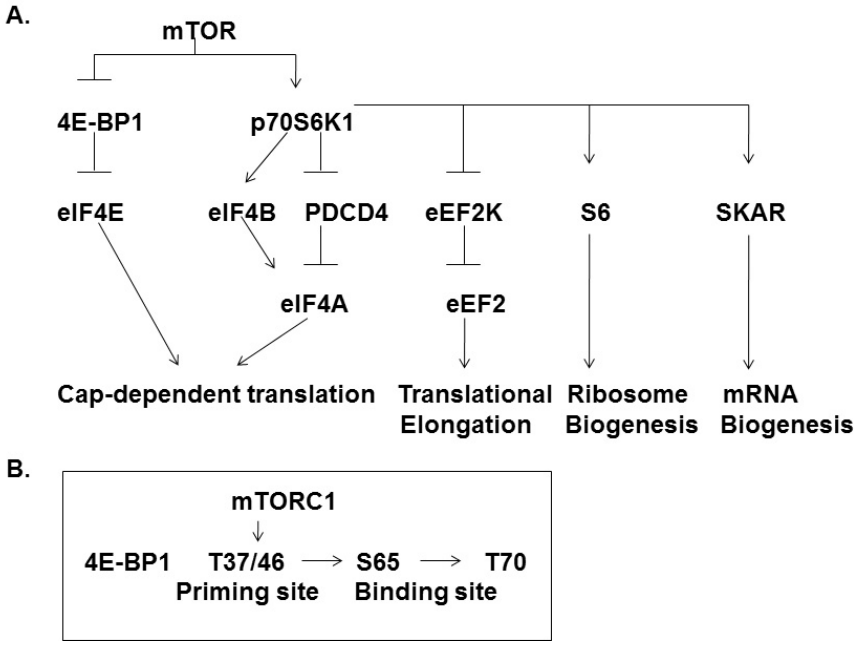

Figure 1.3 mTOR in translational regulation based on a figure from [29]**,** see text for details.

mTOR plays a critical role in translational regulation. Under most circumstances, the rate-limiting step in mammalian translation initiation is the binding of the ribosome to mRNA. Almost all of the factors involved in recruiting the ribosome, including eIF4E, eIF4B, and eIF4G, are phosphoproteins whose phosphorylation states are directly proportional to the translation and growth rates of the cell. In addition, the repressor proteins, 4E-BPs, are similarly phosphorylated under the same circumstances. Thus, increased phosphorylation of these factors in response to numerous extracellular stimuli correlates with

released eIF4E, which enhances translation initiation rates and consequently increases translation of a subset of mRNAs.

1.11 Two classes of translation models: complex 2nd structure vs. simple 5' UTR mRNA

Sonenberg and his colleagues proposed that the eIF4F complex functions to recognize the mRNA 5′ cap and unwind the mRNA 5'secondary structure. It has been postulated that the translation of mRNAs containing extensive secondary structure would be preferably stimulated by increased eIF4E activity [65]. eIF4E overexpression in cells enabled efficient translation of a reporter mRNA in which more secondary structure had been inserted in the mRNA 5'UTR . Indeed, eIF4E overexpression preferentially enhances the translation of mRNAs with structured 5′UTRs [66]. In contrary, dominant–negative eIF4A mutant proteins preferentially inhibit translation of these poorly translated mRNAs in vivo [67]. Additionally, eIF4B inactivation in yeast preferentially inhibits translation of reporter mRNAs possessing long and structured 5' UTRs [68]. Subsequently, several groups identified mRNAs whose translation was preferentially stimulated in eIF4E-overexpressing NIH-3T3 cells as well as other cell lines. These mRNAs include, among others, ornithine decarboxylase (ODC), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF). Two common features of these mRNAs are (1) a relatively long and structured 5'UTR, and (2) most importantly, their protein products function in controlling cell growth and proliferation. Hence, the translational activation of these mRNAs is expected to

promote cell growth and proliferation. ODC has been studied in some detail, as it is a model par excellence for studying translational control by eIF4E. It contains a G/C-rich 5'UTR of ~300 nt and is not well translated in vivo or in vitro. In response to insulin stimulation, which activates eIF4E, its translation increases by ~30-fold [66]. Consistent with these findings, the translation of ODC mRNA in eIF4E-overexpressing NIH-3T3 cells is also increased by ~30-fold [67]. Experimentally induced elevation in the levels of other components of eIF4F and eIF4B would be expected to elicit similar effects.

Recently Sabatini, et al [69] proposed an alternative model for the mRNA features and mechanisms that confer mTORC1-dependent translation control. In this model, the main class of mRNA molecules affected shortly after drug treatment contain short, unstructured 5´UTRs, in contrast to the highly structured 5´UTRs previously reported. They used high-resolution transcriptome-scale ribosome profiling to monitor translation in mouse cells acutely treated with the mTOR inhibitor Torin 1, which, unlike rapamycin, fully inhibits mTORC1. They showed that the subset of mRNAs that are specifically regulated by mTORC1 consists almost entirely of transcripts with established 5′ terminal oligopyrimidine (TOP) motifs, previously unrecognized TOP, or related TOP-like motifs. They found no evidence to support proposals that mTORC1 preferentially regulates mRNAs with increased 5′ untranslated region length or complexity. Rather, they mentioned that loss of just the 4E-BP family of translational repressors, the best characterized mTORC1 substrates, is sufficient to render TOP and TOP-like mRNA translation resistant to Torin 1. Further, the 4E-BPs inhibit translation

initiation by interfering with the interaction between the cap-binding protein eIF4E and eIF4G1. Loss of this interaction diminishes the capacity of eIF4E to bind TOP and TOP-like mRNAs much more than other mRNAs, during this translational program controlled by mTORC1, 4E-BPs and eIF4G1 are its master effectors [69].

Whether mTOR regulates Aurora A translation and by which of the above two models was addressed in this thesis.

1.12 mTOR regulation of protein destruction

mTOR, through regulating ubiquitin/proteasome activity, can modulate protein degradation. This has been noticed in several cases. For instance, mTORC2 targets Akt1 for protein degradation. Wu, *et al* showed that abolishing Akt Ser-473 phosphorylation stabilizes Akt following agonist stimulation [70]. The Akt Ser-473 phosphorylation promotes a Lys-48-linked polyubiquitination of Akt, resulting in its rapid proteasomal degradation. Blockade of this proteasomal degradation pathway with a proteasome inhibitor prolongs agonist-induced Akt activation. mTORC2 first stabilizes Akt protein folding through the Thr450 turn motif phosphorylation and then by promoting Akt protein degradation through the Ser473 hydrophobic motif phosphorylation, thus regulating the Akt protein life cycle [71, 72].

mTOR also regulates cyclin D degradation. Rapamycin induces the ubiquitin-dependent APC system degradation of cyclin D1, accelerating the turnover of cyclin D1, which in turn reduces cyclin dependent kinase (CDK)

activation. This leads to depletion of active CDK4/cyclin D1 complexes, which may cause G1phase arrest. [73]**.** Whether mTOR affects Aurora A protein stability was examined in chapter 4 of this thesis.

1.13 mTOR kinase suppression of PP2A protein phosphatase

In *Saccharomyces cerevisiae*, TOR-mediated signaling activity promotes the interaction of phosphatase-interacting protein Tap42 with protein phosphatase 2A (PP2A) and 2A-like protein phosphatases. The distinct complexes formed between Tap42 and different phosphatases mediate various cellular events and modulate phosphorylation levels of many downstream factors in the TOR pathway in a TOR- and rapamycin-sensitive manner. Cell cycle-dependent distribution of actin is an example [74].

In MDA-MB-231 human breast cancer cells, mTOR-dependent suppression of PP2A is critical for phospholipase D survival signals. Elevated phospholipase D activity suppresses the activity of the putative tumor suppressor PP2A in an mTOR-dependent manner [75].

1.14 Cell cycle

The cell cycle is an ordered set of events, allowing inheritance of the genetic material from mother to daughter cells. It consists of four phases: G1 (cell growth), S (DNA synthesis), G2 (rapid cell growth and protein synthesis), and M phase (the chromosome separation, mitosis, and the cell division into two daughter cells, cytokinesis). Among these phases, mitosis is the most rapid and highly complex process. Within 1-2 hours, five mitosis phase; prophase, prometaphase, metaphase, anaphase and telophase, are completed. These processes are tightly controlled by several kinase families including Aurora kinases, cyclin-dependent protein kinases, and Polo-like kinases (Plk). Errors in the choreography of these processes can lead to aneuploidy or genetic instability, fostering cell death or coursing cancer. Thus, study of the mechanism(s) of mitosis regulation, particular its kinase proteins, may provide information to better understand cancer.

1.15 mTOR in G1 phase of the cell cycle

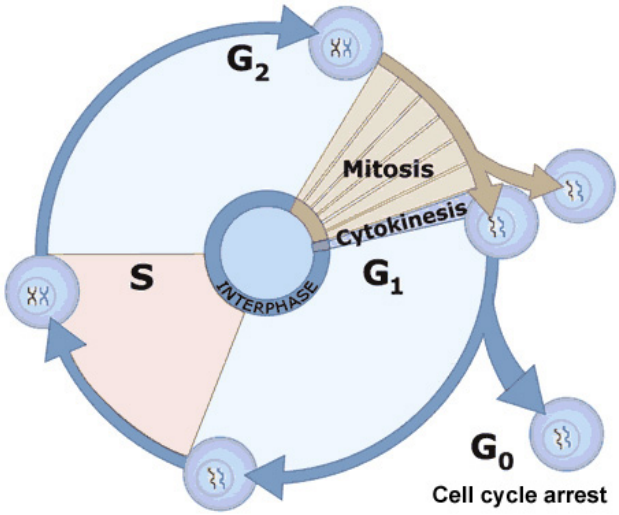

Figure 1.4 Cell cycle based on a figure from Clinical Tools, Inc.

Cell cycle includes phases G1, S, G2, and M (the nuclear division, mitosis and the cell division, cytokinesis). In the presence of unfavorable growth condition, cells may pause for extended periods in G1, or enter a prolonged non-dividing state, G0.

1.15 mTOR in G1 phase of the cell cycle

It is well-documented that mTOR signaling mediates G1 phase progression through regulation of gene transcription, mRNA translation, and protein destruction for certain genes such as $p27^{kip}$, cyclin D1, and CDK4 in many cell types including human ovarian cancer cells [73, 76, 77] [78, 79].

1.16 TOR in G2/Mitosis progression

Studies, primarily on lower eukaryotes, also suggested a role for mTOR in insulin delays the progression of fly cells through G2/M by activating G2/M progression. In fission yeast, increased nutrient provision transiently delays mitotic onset and blocks cell division, which relies upon TOR [80]. In Drosophila, the dTOR/dRaptor complex. dRaptor was found to be required for proper mitotic spindle assembly in Drosophila S2 cells. Mitosis specific raptor phosphorylation is required for normal cell growth and progression through G2/M of the cell cycle [81]. In mammalian cells, Liu et al.at 2007 first demonstrated that human S6K1 phosphorylation and activity peak in M phase [82]. In oral squamous carcinoma and Hela cells, RT-PCR and immunoblot results showed that the level of mTOR mRNA did not change during the cell cycle, while the expression of p70S6K increased noticeably. Furthermore, activity assays in HeLa cells suggested that the activity of mTOR was maintained at a higher level in phase M than in any other phase [82].

Another example is that the cell-cycle G2-M phase gene Ubiquitin-Conjugating Enzyme E2C (UBE2C) was found overexpressed in various solid tumors. mTOR inhibitor CCI-779 inhibited UBE2C mRNA and protein expression reduction in RNA polymerase II loading to the UBE2C promoter, and attenuation of UBE2C mRNA stability, resulting in the cell-cycle G2/M accumulation [83]. Also, it is reported that Plumbagin, a cancer drug, inhibits cyclin B and Cdk1 phosphorylation and destructs CDC25C protein via Akt mTOR, caused G2/M arrest [84].

1.17 Introduction of Aurora family

The Aurora kinase family is highly conserved among species, from Saccharomyces cerevisiae, Drosophila to Human [85]. Mutations in Aurora of drosophila and related Saccharomyces cerevisiae ploidy level 1 (Ipl1) kinase are known to cause abnormal chromosome segregation [86]. The mammalian Aurora family has three members: Aurora A, B, and C, all of which have a carboxyl terminal ‘‘destruction box’’ (D-box), however, Aurora A uniquely has an amino terminal ‘‘D-box-activating domain box’’ (A-box) [87, 88] required for the functional activation its D-box (Figure 1A). Though the majority of the structure is conserved, Aurora members’ functions are highly variable because of the differences in subcellular distribution and their activation levels during the cell cycle [89].

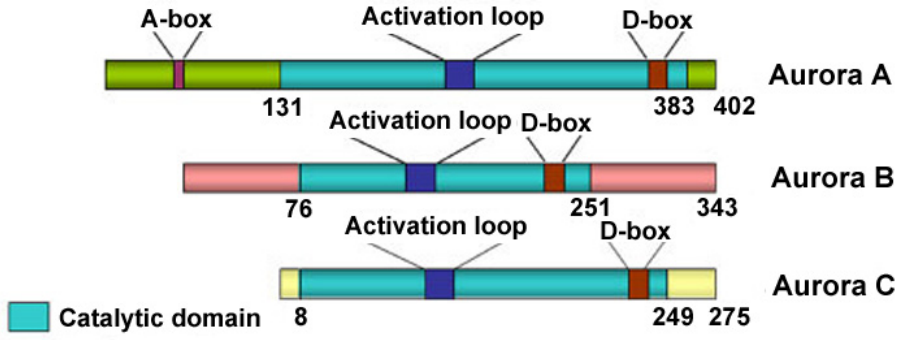

Figure 1.5 Domains of Aurora kinases based on a figure from [88]

Activation loop is required for kinase activity and typically is phosphorylated. A D-box is found in many targets of the anaphase promoting complex including Aurora A, B and C kinases. Aurora A also has an A-box whose phosphorylation status regulates protein destruction [88].

Human Aurora A was originally identified by its close homology to Saccharomyces cerevisiae Ipl1. The latter was discovered in a screen for Saccharomyces cerevisiae mutations affecting chromosomal segregation [55, 56]. Subsequently in 1995, Glover et al., identified the first allele for Aurora A in Drosophila mutants that were defective in spindle-pole behavior. The name Aurora is based on the aurora borealis, a phenomenon of the night sky in the polar region [57]. Human Aurora A, is also known as Aurora2, STK15, BTAK, and AIK. It is cell-cycle regulated, maps to chromosome 20, and is highly expressed in colon and breast and several other tumor types (5–7, 15). The full-length aurora A cDNA contains a 1209-bp open reading frame that encodes 403 amino acids with a predicted molecular mass of 46 kDa. The typical phenotype is monopole spindle, resulting from its failure of centrosome generation and separation in cells.

1.18 Transcription of Aurora A gene

Transcription of the Aurora A gene (AURKA) is cell-cycle regulated. The promoters of the Aurora A gene contain specific elements cell cycle dependent (CDE)/cell cycle genes homology region (CHR), which are responsible for transcription at the G2 phase of the cell cycle [90]. However, the mechanism of transcriptional regulation is not fully understood. It was reported that AURKA mRNA level is mediated by transcription factors belonging to the E-twenty six (Ets) family, including epidermal growth factor (EGF), gamma-aminobutyric-acid receptor epsilon subunit precursor (GABP), and transcription factors responsible for adenovirus E4 gene transcription (E4TF1), which utilize the CDE and CHR promoter elements. AURKA mRNA expression is affected by its promoter interacting with activator-binding proteins, which vary during different stages of the cell cycle [90-92]. Hence, a wide range of variation is observed in the expression and activity of Aurora A during cell cycle progression [86, 91, 93]. In transformed cells which overexpressed EGF, transcription of Aurora A was increased via nuclear translocation of the EGF receptor. EGFR, upon phosphorylation at Thr845, is activated and binds to the promoter region of Aurora A, facilitating its transcription [94]. Together, this evidence provides the possibility that mTOR may regulate Aurora A mRNA expression and it was examined in chapter 3 of my study.

1.19 Translation of Aurora A

The 5' UTR of human AURKA mRNA has six alternative splicing variants that share a common open reading frame that is translated into a single common protein [95] (Figure 1.6).

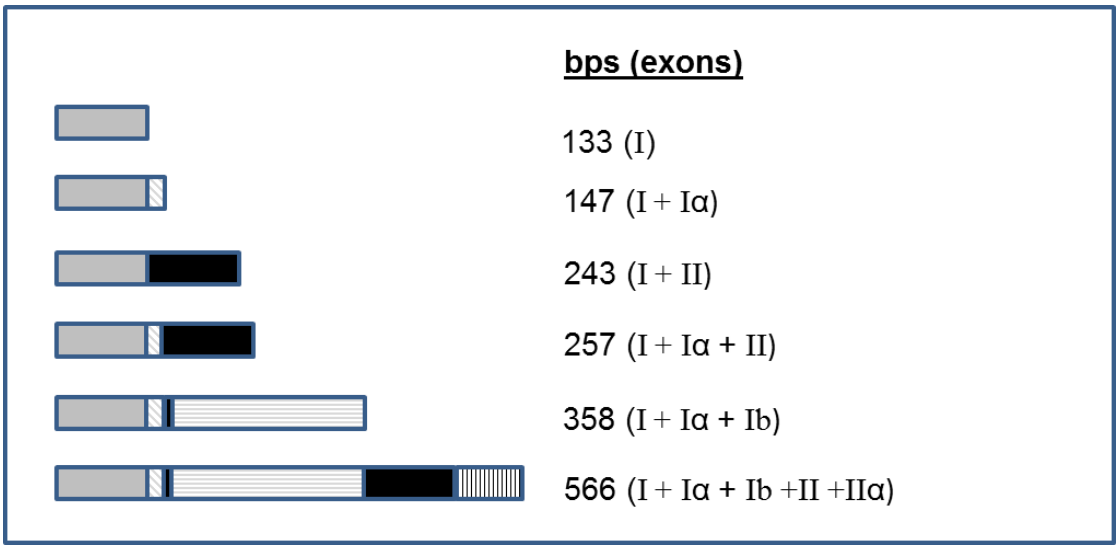

Figure 1.6 Six alternative splicings of AURKA 5' UTR based on a figure from [95]
Among the six variants, variant 1, 3, and 5 contain exon II, and variant 5 reportedly responds to EGFR stimulation [95].

A cDNA representing variant 5 of the AURKA mRNA was cloned from human B cell library [86], the MDA-MB-231 breast cancer cell line [96] and colon cancer cell line [97], while variant 6 was discovered in testis cDNA cloning according to NCBI (National Center for Biotechnology Information) and in mixed and uncharacterized tissues from brain and liver.

A recent study showed that upon stimulation with EGF in colorectal cancer cell lines there was an increased the Aurora-A protein expression [95]. The overexpression of EGFR was associated with higher expression of Aurora A in

clinical colorectal samples. Activation of the PI3K/Akt/mTOR and MEK/ERK pathways mediated the effect of EGF-induced translational up-regulation. Particularly, only the splicing variants containing exon 2 of Aurora A mRNA increased its interaction with the translational complex eIF4F to synthesize Aurora A proteins under EGF stimulus [95].

A more recent report illustrates that human pumilio homology protein 2 (PUM2), an RNA-binding protein and a translational regulator, promotes both protein stability and kinase activity of Aurora A [98]. It was reported to bind to 5' UTRs of mRNA and to repress gene expression by controlling cytoplasmic polyadenylation and affecting mRNA translation. Whether it is regulated by mTOR is unknown.

1.20 Aurora A protein destruction and ubiquitin/proteasome pathway

In both low and high eukaryotes, Aurora A protein began to accumulate in G2 of the cell cycle and was stable throughout mitosis and rapidly disappeared in early G1[99]. Its degradation is mainly controlled by Anaphase-Promoting Complex (APC) ubiquitin/proteasome pathway.

The ubiquitin/proteasome system is a major proteolytic system in the eukaryotic cell for selective destruction of proteins [100, 101]. This system contains an ubiquitin activating enzyme (E1), an ubiquitin conjugating enzyme (E2), and an ubiquitin ligase (E3). The 26S proteasome then degrades the ubiquitinated proteins [102-105].

The common feature of proteasome mediated protein degradation is the covalent attachment of ubiquitin to lysine residue of proteins targeted for degradation. Repeated addition of ubiquitin molecules to the substrate leads to the formation of polyubiquitin chains that are recognized by the 26S proteasome [106, 107]. In contrast to the E1 and E2 enzymes the E3 ubiquitin ligases display substrate specificity. The E3 enzyme for the ubiquitination of several mitotic proteins, including Aurora A, has been reported [108]. Anaphase promoting complex/cyclosome (APC/C), an E3 enzyme, is a large, multi-subunit ubiquitin-protein ligase that catalyzes the attachment of ubiquitin to mitotic proteins, promoting their ubiquitin-dependent proteolysis. In eukaryotes, ubiquitination by APC/C is controlled by activator subunits that bind the APC/C core at different stages of the cell cycle. There are two activators, cell-division cycle protein 20 (Cdc20) and Cdc20 homologue 1 (Cdh1). Cdc20 binds and activates the APC/C,

which triggers metaphase-to-anaphase transition by stimulating the destruction of regulatory proteins such as securin and cyclins that govern these events. While Cdh1 is primarily responsible for maintaining APC/C activity in later mitosis and throughout G1, it ensures some mitotic kinases such as Aurora A protein to be destructed and inactivated. The C-terminal D-box in Aurora-A is required for Cdh1-induced destruction, whereas the N terminal KEN-box is not. Destruction also requires an intact A-box, in which a conserved core consists of amino acids QRVL, a short stretch named D-Box-activating domain (DAD) located to the N-terminus, whose deletion arrests the APC/C/Cdh1-mediated degradation [109-112]. Briefly, APC/C/Cdh1 plays an important role in Aurora A protein stability and whether it is involved in mTOR-mediated Aurora A degradation will be investigated in chapter 3 of this study.

As Cdh1 maintains APC/C activity in G1 phase [113-115] and Aurora A destruction was found in G1cell lysates [116, 117], it suggests that we can use asynthronized cells (~70-80% G1 population) for study degradation of Aurora A protein.

1.21 A-Box and D-Box of Aurora A required for Aurora A destruction

The APC/C/Cdh1 pathway predominantly regulates Aurora A levels at the time of mitotic exit. Full-length Aurora A requires both A-box and D-box for its Cdh1-dependent destruction and dephosphorylation of Ser53 during mitotic exit by protein phosphatase 2A (PP2A). This results in the conformational change in the D-box and in turn makes it more accessible for APC/C-mediated ubiquitination, and further exposes the A box for complete ubiquitination and degradation. Cdh1 recognizes the Arg378 of D-box. APC/C is more abundant during mitotic exit, which then targets Aurora A via its A-box and D-box sequence to the ubiquitin-dependent proteasomal degradation, as reported earlier [109, 117-119].

1.22 Co-localization and interaction of Aurora A and PP2A

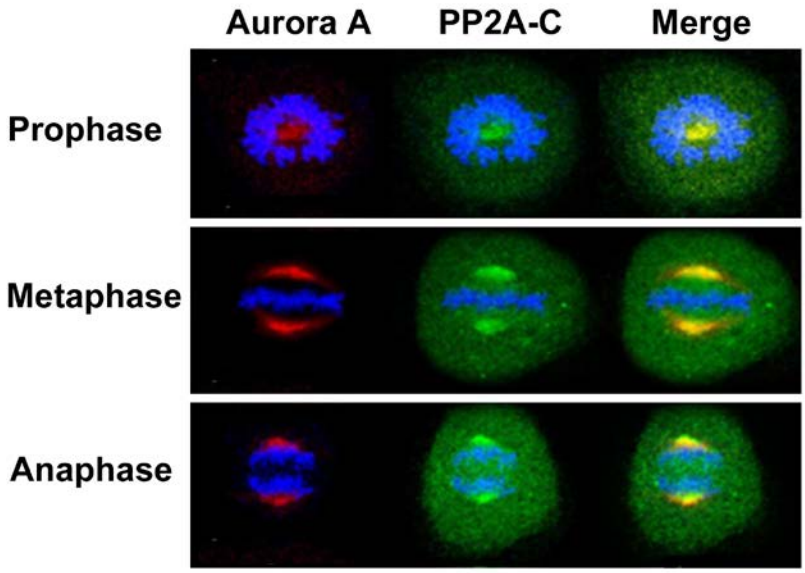

Figure 1.7 Co-localization of Aurora A with PP2A-C at centrosomes during mitosis [119].

PP2A co-localized with Aurora A in prophase, metaphase, and anaphase cells. Aurora A is shown in red and PP2A catalytic subunit in green [119].

The protein phosphatase PP2A is a member of the serine/threonine PP2 phosphatase family. PP2A is a hetero-trimeric complex composed of three subunits: catalytic C subunit, structure A subunit, and regulatory B subunit. The former two subunits form a core dimer and interact with one of the several B-type regulatory proteins (B, B', and B''), which regulate the core dimer activity and substrate's specificity. It was reported that PP2A affects numerous cellular functions, including signal transduction and cell cycle control. It was noticed that PP2A plays important roles in G2/M transition and mitosis completion. PP2A has also been proposed to play a role in the metaphase-anaphase transition in both

yeast and mammalian cells. Recently, Andersen *et al* demonstrated that PP2A localizes to interphase centrosomes [119], which is known to be critical for mitosis initiation and progression, and the centrosomes contain key mitotic kinases such as Aurora A [88]. Importantly, PP2A physically interacts with Aurora A during mitosis in human cells and is inhibited by a phosphomimetic peptide containing S51 in the A-box of the Aurora A. Therefore PP2A appears to regulate Aurora A protein stability. In chapter 3, I will address whether mTOR regulates Aurora A via this phosphatase.

1.23 Aurora A in mitosis of the cell cycle

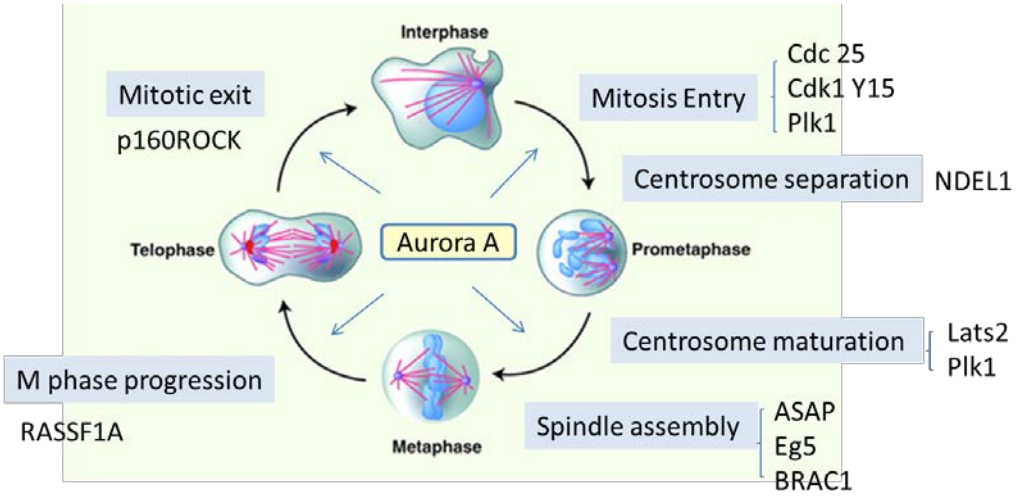

Figure 1.8 Aurora A in mitosis of the cell cycle based on a figure from [120]

Aurora A facilitates mitotic entry and is essential for centrosome separation and bipolar spindle formation. Aurora A is also involved in the process of mitotic exit during the cell cycle [88].

Aurora A kinase is intimately involved in several essential steps of the cell cycle, such as mitotic entry, centrosome duplication, separation, and maturation [121] as well as bipolar spindle assembly [122], chromosomal alignment, cytokinesis, and mitotic exit [88]. Dysregulation of Aurora A expression and activity leads to centrosomal as well as spindle assembly checkpoint defects, aneuploidy, genetic instability, transformation, and tumorigenesis.

Mitotic entry: One substrate of aurora A is Cdc25B, a direct regulator of the cyclin B1-Cdk1 complex, which provides the basis for the role this enzyme plays in regulating entry into mitosis [123]. Aurora A regulates centrosome maturation by moderating the recruitment of proteins, such as TPX-2, Ajuba,

Bora and Lats, which are essential for accumulating microtubule spindle components, such as gamma-tubulin. Aurora A is also associated with separation of centrosomes, through phosphorylation of the kinesin motor protein, Eg5. In addition, this enzyme regulates the microtubule network that forms mitotic spindles, through regulation of the EXTAH multiprotein complex. One of the characteristic features of Aurora A inhibition is monopolar spindle formation and cell cycle arrest [122].

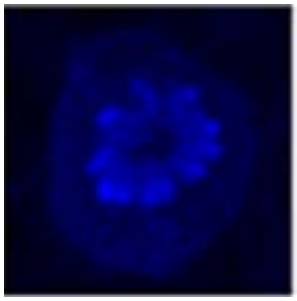

Figure 1.9 Cell lacking Aurora A forms a monopolar chromosome [122]

Image of murine embryonic fibroblast cells following knockout of AURKA shows a surrounding circular chromosome array, called monopolar chromosome. The chromosomal DNA was stained with DAPI [122].

1.24 Aurora A in tumorgenesis

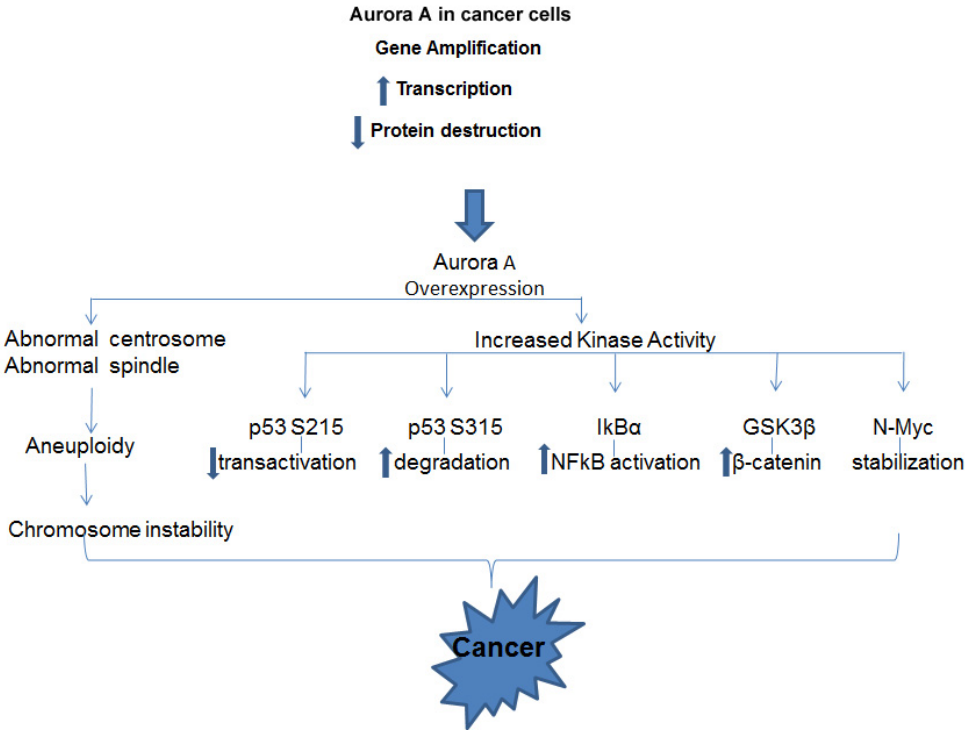

Figure 1.10 Aurora A in tumorigenesis based on a figure from [88]

Aurora A is overexpressed in many cancers by several mechanisms, including gene amplification, elevated transcription as well as suppression of protein degradation. Overexpression of Aurora A leads to abnormal spindle formation and cytokinesis failure. Polymorphism of the Aurora A gene can also contribute to tumorigenesis by abrogation of its normal mitotic regulatory functions. Aberrant phosphorylation of the kinase substrates by hyperactive Aurora A are also a probable cause of carcinogenesis [88].

Aurora A is overexpressed in cancers by several mechanisms, such as gene amplification, enhanced transcription, as well as suppression of protein degradation. Overexpression of Aurora A leads to abnormal spindle formation and cytokinesis failure. Subsequently, aneuploidy succeeds polyploidy which, in the absence of a functional p53-Rb pathway, results in amplification of centrosome number and chromosome instability. Polymorphism of Aurora A gene can also contribute to tumorigenesis by abrogation of its normal mitotic regulatory functions. Aberrant phosphorylation of substrates by hyperactive Aurora A could also be a probable cause of carcinogenesis [88].

Aurora A acts as an oncogene by phosphorylating various substrates. Most of these are associated with either centrosome associated or spindle assembly related protein. The approximately 26 known substrates of Aurora A protein kinase include: Centrosomin, CENP-A, p53, IkBa, cdc25B, Plk1, and Eg5 [88].

CDC25B: As early as 2000, cell division cycle 25 homolog B (Cdc25B), a CDC25 phosphatase, was identified as a substrate of Aurora A. Aurora A phosphorylates Cdc25B at Ser353, which then localizes at the centrosome. Aurora A recruits Cdk1/Cyclin B1 to the centrosome prior to onset of mitotic events. So Aurora A is upstream of the Cdc25B phosphatase as well as the Cdk1/Cyclin B1 complex for commitment of mitotic entry [88] [124].

CENP-A: Aurora A phosphorylates centromere protein A (CENP-A) at the amino-terminal serine 7. This phosphorylation is important for the proper

attachment of microtubules to the kinetochore and consequently for chromosome alignment and segregation [94, 125]

Plk: Polo-like kinase is another mitotic kinase that localizes to the centrosome. It has functions that are quite similar to Aurora A that aid in bipolar spindle formation. In the presence of Aurora A-binding protein, Bora, Plk1 activation occurs upon phosphorylation of Thr210. Phosphorylation of this site is mediated by Aurora A during G2/M transition, and maintained until cells enter into mitosis [88, 126].

p53, a well-known protein 53 tumor repressor, is also a substrate of Aurora A. There are two Aurora A phosphorylation sites identified on p53. Aurora A directly phosphorylates p53 at Ser315, which augments p53 and Mdm2 interaction. This facilitates ubiquitination and mdm2-mediated degradation of p53 in cancer cells [127]. Another Aurora A phosphorylation site is Ser215, within p53's DNA-binding domain. Phosphorylation of Ser215 by Aurora A enables inactivation of p53's by DNA-binding and transactivation activity. As a result, p53 target genes p21 and PTEN are down-regulated, and p53 tumor suppressor activity is inhibited by Aurora A [88, 127-129]

IkBα: an nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha, similarly Aurora A kinase-mediated IkBα phosphorylation determines the stability of this protein. Upon phosphorylation of the residues Ser32 and Ser36, IkBα gets degraded leading to the activation of NFkB. As a consequence, NFkB is free to translocate to the nucleus and thus can activate transcription of is downstream targets [88, 130].

BRCA1: Potential roles of Aurora A in cell transformation were also demonstrated from recent studies that this kinase phosphorylates a breast cancer tumor suppressor BRCA1 at Ser308 [14]. Both proteins are localized on the centrosome at the beginning of mitosis [15], suggesting that signaling between these two proteins are crucial for regulation of a normal cell cycle.

Aurora A is overexpressed in a wide range of human cancers. It maps to chromosome 20q13.2, a region that is frequently mutated in human cancers. There are numerous reports showing significant incidence of Aurora A amplification and overexpression in human breast, bladder, ovarian, colon, and pancreatic cancers.

There are several mechanisms by which Aurora A promotes tumorigenesis: Aurora A overexpression causes checkpoint defect and genome instability; Aurora A overexpression in polyploidy is linked to cancer; Aurora A overexpression results in extra copies of centrosomes in cell lines as well as in rat mammary models. However, centrosome amplification observed in the cells overexpressing Aurora A was not due to centrosome duplication, but as a consequence of cytokinesis failure and multinucleation. Disruption of the normal centrosome duplication cycle results in the formation of monopolar or multipolar spindles leading to polyploidy, which is a precursor of aneuploidy and contributes to carcinogenesis.[88].

In summary, mTOR-regulated protein expression potentially impacts G2/M progression. Since Aurora A is an important mitotic kinase that plays crucial roles

in mitotic progression and tumorigenesis it has been examined in this thesis as a potential mediator of mTOR action.

RESEARCH OBJECTIVES

mTOR is a key regulator of protein synthesis that is inhibited by the macrolide antibiotic, rapamycin. Rapamycin has been shown to cause arrest of cells in G1 phase of the cell cycle. However, whether mTOR regulates mitosis is uncertain. Since G2/mitosis progressions are important for cell division, I will focus on whether mTOR regulates the expression and/or activity of key proteins that control G2/mitosis. These studies may provide a better understanding of how mTOR coordinates cell cycle progression and protein synthesis for cell growth and proliferation.

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

Rapamycin (FRAP/mTOR inhibitor) from Cell Signaling. Torin 1 from TOCRIS; Ku-0063794 (KU63794) from Selleckchem; PP242 from BioVision; Proteasome inhibitor MG132 10 mM stocks in dimethyl sulfoxide (DMSO) were purchased from Calbiochem; okadaic acid (OA) from Sigma. Plasmids pcDNA3.1-Flag-Aurora A S51A and Flag-Aurora A S51D were gifts from Takashi Takata in Japan.

2.2 Cell culture and cell cycle synchronization

HeLa cells were obtained from ATCC and cultured in RPMI-1640 supplement with 25 mM L-Glutamine and 10% fetal bovine serum (FBS) (Hyclone). HEK293T cells were cultured in DMEM (BioWhittaker) supplemented with 10% FBS. The cells were maintained at 37°C with 5% CO_2.

For synchronization, double thymidine blockage/release approach was employed [86]. HeLa cells were seeded at 100,000 cells per well in a 6-well plate. The following day, thymidine (Sigma) was added to the media to a final concentration of 2.5 mM and the plates were incubated for 16 hours at 37°C. The plates were then washed three times with phosphate-buffered saline (PBS) and normal growth medium was added. Following 8 hours at 37°C, cells were treated with thymidine for additional 16 hours. To release them from the drug treatment, cells were washed with PBS three times and normal growth medium was added. For monitoring mTOR effects on G2/M phases, mTOR inhibitor KU63794 1μM was administrated at indicated times.

2.3 Cell lysates preparation and immunoblotting analysis

HeLa cells were lysed in 50 mM HEPES, pH 7.5, 500 mM NaCl, 6 mM $MgCl_2$, 0.2 mM Na_3VO_4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.05% SDS, 19 µg/ml aprotinin, and1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged at 10,000 rpm at 4^oC for 10 minutes and the soluble components were then collected into 1.5-ml tubes. Protein concentrations were determined using a Bio-Rad protein quantitation kit according to the manufacturer's instructions. Equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis using 4-20% gradient Tris-Glycine gels (Invitrogen) and proteins were then transferred to PVDF-FL membranes (Millipore). Transferred membranes were then incubated in TBS-T solution containing 20 mM Tris-HCl (pH 7.9), 150 mM NaCl, and 0.2%Tween 20 supplemented with 5% nonfat milk, followed by incubation with the primary antibody specific to indicated protein. Antibodies specific to Aurora A/AIK(1G4) were obtained from Cell Signaling, glyceraldehyde-3-P dehydrogenase (GAPDH) from Biodesign, M2 anti-FLAG from Sigma, Histone H3 and Histone H3 Ser10 from Millipore, S6K1 and S6K1 Thr389, 4E-BP1 and 4E-BP1 Thr37/46, Ser65, and Thr70, Cdc2 (Cdk1) were from Cell Signaling. The membranes were incubated in TBS-T containing 5% nonfat milk over night at 4^oC. The membranes were then washed three times in TBS-T followed by incubation with horseradish peroxidase-labeled secondary antibody. Using SuperSignal West Femto Maximum Sensitivity substrate (Thermo Scientific), immunoblots were visualized by exposing membranes to x-ray film.

2.4 Flow cytometry

HeLa cells growing in cultures were washed, trypsinized and harvested in PBS and fixed in 70% ethanol overnight at 20^{o}C. After treatment with 1 mg/ml RNase A (Sigma) at 4^{o}C for 60 minutes, DNA staining was performed using propidium iodide (Invitrogen) in the dark, prior to flow cytometric analysis. Analysis was performed on a FACS flow cytometer (Becton Dickinson, USA). For each sample, 25,000 events were collected and aggregated cells were gated out. The percentage of cells existing within the different phases of the cell cycle was determined using CellQuest Pro software.

2.5 RNA isolation and quantitative real-time-PCR

Total cellular RNA was prepared using TRIzol reagent (Invitrogen) according to the instruction manual. Cells were lysed directly in the culture dish. Samples were incubated for 5 minutes in TRIzol at room temperature. Chloroform 0.2 ml was added for every 1 ml of TRIzol used. Samples were shaken vigorously for 15 seconds and incubated at room temperature for 2-3 minutes. Samples were centrifuged for 15 minutes. at 12,000 x g at 4°C. The RNAs were precipitated by mixing with 0.5 ml of isopropanol for each 1 ml of TRIzol used. Samples were incubated at room temperature for 10 minutes and centrifuged for 10 minutes at 12,000 x g at 4°C. Then pellets were washed with 1 ml of 75% ethanol and the RNAs were dissolved in RNase free H_2O.

The first strand cDNAs were synthesized used a high capacity cDNA reverse transcription kit (AppliedBiosystems) according to the instruction manual.

RT-PCR mixtures were prepared by using TaqMan® Gene Expression Master Mix (Appliedbiosystems) with a probe specific to human cyclin B1 or AURKA (AppliedBiosystems, Hs01582072_m1, NM_003600), and GAPDH (AppliedBiosystem, Hs02758991_g1) was used as a control. The RT-PCR reaction and analysis were performed using a 7900HT system (AplliedBiosystem).

2.6 Cap-binding assay

For the affinity purification of eukaryotic mRNA cap-binding proteins, Sepharose 4B and 7-Methyl GTP-Sepharose 4B were obtained from GE Health Sciences. After washing with PBS, 1.5×10^6 cells were lysed in 1 ml of lysis buffer (50 mM HEPES at pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM Na_3VO_4, 25 mM glycerophosphate, complete mini protease inhibitor cocktail [Roche], and 0.5% NP-40) and extracts clarified by centrifugation at 10,000 × g for 10 minutes, at 4°C. Supernatants pre-cleared with Sepharose 4B 30 µl for 20 minutes were next incubated with 7-Methyl GTP-Sepharose 4B 30 µl for 2 hours at 4°C. After incubation, the beads were spun down at 2000 rpm for 5 minutes and washed three times with lysis buffer, denatured by the addition of 50 µl sample buffer and boiled at 100^oC for 5 minutes, and the bound proteins were analyzed by SDS-PAGE and immunoblotting [131]. The protein levels were quantitated by phosphoimager software.

2.7 Rapid Amplification of cDNA Ends (5' RACE)

In mammalian cells, there are 6 transcriptional splicing variants of Aurora A. In order to know which version existed in HeLa cells in this study, we carried out a 5'RACE assay (Invitrogen) according to the manufacturer's instructions. The total RNA was obtained from the kit. First strand cDNA was synthesized from total RNA using a gene-specific primer Aurora-A 5'UTR reverse R1, 5'-CAGTTTTCTTTAGATCGGTCC and M-MLV RT (a derivative of moloney murine leukemia virus reverse transcriptase). After the first strand cDNA synthesis, the original mRNA template was removed by treatment with the RNase Mix. Unincorporated dNTPs, primer AURKA R1, and proteins were separated from the cDNA using a S.N.A.P. Column. A homopolymeric tail was then added to the 3'-end of the cDNA using terminal deoxynucleotidyl transferase and dCTP. Tailed cDNAs were then amplified by PCR using a mixture of three primers: a nested primer reverse AURKA R2, 5'-GAAAATGCTGGGATTACGGG-3', which annealed 3' to the AURKA R1; and a combination of a complementary homopolymer-containing anchor primer and corresponding adapter primer which permit amplification from the homopolymeric tail. Following an initial denaturation step at 94 °C for 3 minutes, 35 cycles of 30 s at 94 °C, 1 minute at 55 °C, and 1 minute at 72 °C were run. The final extension step at 72 °C was carried out for 10 minutes. The 5′-RACE products were then analyzed using electrophoresis on a 2 % agarose gel. The prominent DNA bands were excised, gel purified and sub-cloned into pCR 2.1vector (TA Cloning Kit, Invitrogen) and sequenced. The

transcriptional start site was determined as the first nucleotide that is 3’ to the adapter sequence ligated to the 5’of the mRNA transcripts.

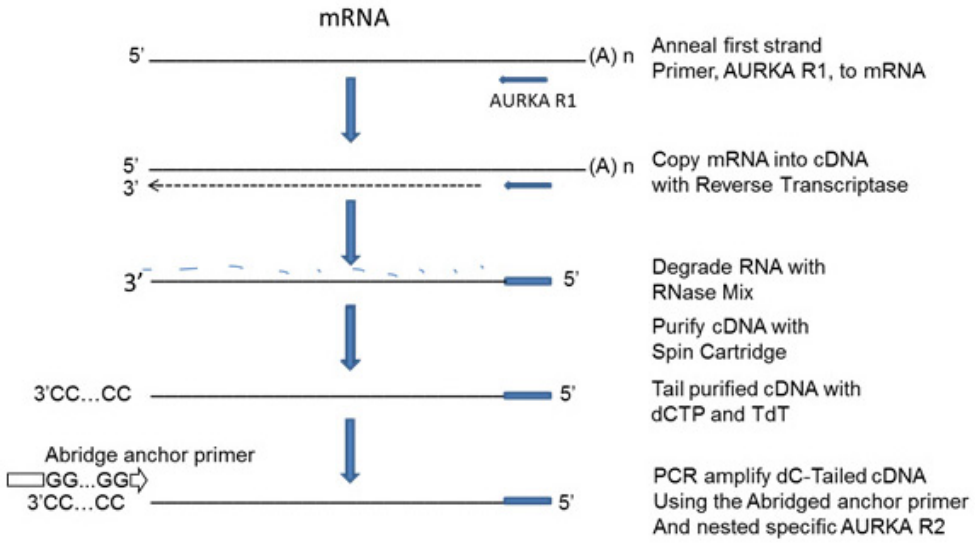

Figure 2.1 Overview of the 5' RACE procedure based on a figure from Invitrogen (5' RACE version 2.0)

First strand cDNA is synthesized from total RNA using a gene-specific primer AURKA R1. After first strand cDNA synthesis, the original mRNA template is removed by treatment with the RNase Mix. The cDNA is purified with a spin cartridge. A homopolymeric tail is then added to the 3'-end of the cDNA. PCR amplification is conducted with a nested, gene-specific primer R2. Following amplification, 5' RACE products can be cloned into the pCR 2.1 vector for sequencing (Invitrogen 5' RACE version 2)

2.8 Lentiviral production and transduction for knock down

Mission shRNAs FZR1/cell division cycle 20 related 1(Cdh1) were obtained from Sigma as follows:

Sigma cat #	Sequence
TRCN0000010856 NM_016263.x-457s1c1	CCGGCGGCAACGATGTGTCTCCCTACTCGAGTAGGGAGACACATCGTTGCCGTTTTT
TRCN0000231901 NM_016263.2-209s21c1	CCGGGTGAACTTCCACAGGATTAACCTCGAGGTTAATCCTGTGGAAGTTCACTTTTTG
TRCN0000231902 NM_016263.2-405s21c1	CCGGAGAAGGGTCTGTTCACGTATTCTCGAGAATACGTGAACAGACCCTTCTTTTTTG
TRCN0000231903 NM_016263.2-1434s21c1	CCGGTGAGGTTCTGGAACGTCTTTACTCGAGTAAAGACGTTCCAGAACCTCATTTTTG
TRCN0000231904 NM_016263.2-1498s21c1	CCGGCTTCACCAGGATCCGGTAAACCTCGAGGTTTACCGGATCCTGGTGAAGTTTTTG

Table 2.1 FZR1/Cdh1 shRNA sequences.

These shRNAs target different sites of coding region of Cdh1 mRNA.

To produce lenti-viral shRNA, HEK293T cells were plated at $6x10^5$ cells/well in a 6-well plate. At next day the cells were transfected with 1µg Lenti-viral constructs containing shRNAs and 4.6 µl lentiviral packaging mix (Sigma) with Fugene 6 (Roche) according to the manufacturer's instructions [132]. At 48 and 72 hours post transfection, the supernatants containing the virus were harvested and centrifuged at 15,000 rpm for 3 minutes. The pellets were discarded and the supernatants were stored at -80^oC until needed.

To knock down the endogenous FRZ1 gene, HeLa cells were plated at $1.5X10^5$ cells/well in a 6-well plate. At next day, the cells were transduced with the Lenti-viral shRNAs 0.5 ml and 8 µg/ml polybrene. At 72 hours post transduction, the cells were lysed and knockdown level of Cdh1proteins (FZR1 products) were analyzed by immunoblotting assay.

2.9 RNAi and transfection

Commercial pool siRNA duplexes targeting to Rheb were obtained from Dharmacon/Thermo Biosciences. HeLa cells were plated at 1.5 x 10^5 cells/well in a 6-well plate and incubated for 24 hours before siRNA transfection. Oligofectamine reagent (Invitrogen) was used to transfect siRNA into the cells, basically following the recommended procedures by Invitrogen. Typically 50 nM siRNA oligos along with 4 µl oligofectamine reagent for HeLa cells were used per transfection. Protein lysates were harvested 48 hours after transfection to analyze knockdown levels of Cdh1 proteins by immunoblotting as described previously.

2.10 Luciferase assay

The 133-bp of AURKA variant 6 and the 243-bp variant 5 (see the sequences below) were synthesized by gene synthesis (GenScript), with Kas I and HindIII restriction sites at the 5'and 3' ends, respectively. Synthesized fragments were cloned into TK-Luc vector [133] at corresponding restriction sites. The isolated clones were confirmed by sequencing. Plasmid transfections were performed using HeLa cells grown to 40% confluence and the FuGENE 6 transfection reagent (Roche Applied Science). The transfections were carried out in triplicates using TK-AURKA variant 5-Luc or TK-AURKA variant 6-Luc plasmids, with TK-Luc plasmids serving as an internal control (Promega). 24 hours post transfection, KU63794 1 µM was added to the cell medium. After 2 hours mTOR inhibitor treatment, firefly luciferase activity was measured by

Envision with value of relative light units. Results are presented as means +/- SD that were derived from three independent experiments. Parallel to the luciferase assays, the amount of firefly luciferase mRNA in each transfected condition was measured by qRT-PCR method. Efficiency of mTOR inhibition by KU63794 was examined by immunoblotting assay as described previously.

2.11 Rac1 activity assay

Transfected HeLa cells were washed twice in ice cold phosphate buffered saline (PBS), and lysed in 1 ml of lysis buffer containing 50 mM Tris HCl pH 7.4, 10% glycerol, 200 mM NaCl, 2.5 mM MgCl2, 1% IGEPAL (Sigma) and protease inhibitors 1 mM p-methylsulfonylfluoride (PMSF) and 0.05 trypsin inhibitor units/ml of Aprotinin at 4°C for 5 minutes. The cellular extracts were harvested and centrifuged at 14,000 rpm for 5 minutes. Eight hundreds µl of the cleared lysate were then rocked with 30 µl of GST-PAK-RBD-bound beads for 45 minutes at 4°C to pull down GTP bound Rac1. The beads were then washed three times with the above described buffer. Rac1 was detected by running the samples on SDS page gels and immunoblotting with the Rac1 specific antibody [134, 135].

2.12 Chemotaxis assay

Following siRNA treatment for 48 hours, U373MG cells were detached and loaded in the upper portion of the chamber, allowing cells to move through a permeable filter toward 0.1% Fetal Bovine Serum (FBS) present in the lower portion of the chamber [136] [137, 138]. After 16 hours, non-migrating cells on the upper filter surface were removed with a cotton swab, and migrated cells on the bottom of the filter were fixed with 10% neutral buffered formalin for 10 minutes and stained with crystal violet dye. The mounted filter membranes were photographed with a microscope equipped with a camera at 1x100 magnification and 5 fields were quantified for each slide. The data presented in this study represent three independent experiments

2.13 Statistical analysis

Data were expressed as mean and standard error of the mean. Statistical analysis was performed with a two-tailed unpaired student t-test. A P-value of < 0.05 was considered to be statistically significant. All calculations were performed on the means of triplicate measurements of at least three independent experiments.

CHAPTER 3 RESULTS

Rheb/mTOR Pathway Regulates Cell Migration

3.1 Rheb regulates ribosomal S6 activation in U373 glioblastoma tumor cells.

Rheb is highly expressed in the brain; S6 phosphorylation level is elevated in PTEN-deficient glioblastoma brain tumor samples (Lawrence Quilliam, et al. unpublished), so I anticipated that the PI3K/Rheb/mTOR pathway would be functioning in U373MG, a PTEN-deficient human glioblastoma cell line, and if so, blocking Rheb expression would block the important PI3K/mTOR signaling to activate S6. To test this notion, I first determined whether the Rheb protein is expressed in cultured U373MG cells and we then examined if knocking down Rheb expression can reduce phosphorylation of ribosomal protein S6 downstream of mTOR. As expected, the level of Rheb protein is readily detected in U373MG cells. Notably, suppressing Rheb with siRNA reduced the S6 phosphorylation level (Figure 3.1), suggesting that Rheb lies upstream of the mTOR pathway in U373MG cells.

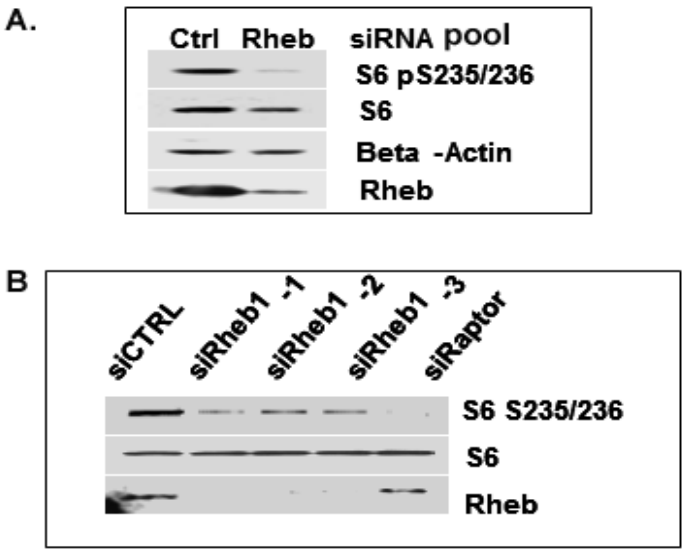

Figure 3.1 Knocking down Rheb reduces ribosomal S6 phosphorylation

U373MG were transfected with 50 nM of short interfering (si) RNAs, either non-targeting 2 (control) or Rheb. After 48 hours, the cells were lysed and equal amounts of the proteins were analyzed by immunoblotting using specific antibodies to Rheb and phosphorylation of S235/236 residues of ribosomal S6 protein, and total S6 protein level served as a loading control.
A. siRNA pool is shown in A and three unique Rheb siRNAs to Rheb in B. siCTRL was a control, which represents a sequence not present in human or mouse genomes. A siRNA to the mTORC1 component, Raptor, was used as a positive control.

3.2 Knocking down Rheb induces F-actin reorganization and elevates Rac1-GTP levels

Interestingly, suppressing Rheb expression in the U373MG glioblastoma cell line also was found to change cell morphology and cytoskeletal organization. F-actin staining with fluorescently-tagged phalloidin showed actin polymerization at the leading edge of the cells in fan assemblies, or lamellipodia following depletion of Rheb expression (Figure.3.2A). Since lamellipodia are induced by the Rac1 GTPase, I next asked if Rac1 is activated after knocking down Rheb. Pulling down Rac1 with GST-RBD-PAK (a GST fusion protein containing the Rac1-GTP-binding domain of p21 Rac1/Cdc42-activated protein kinase, immobilized on glutathione agarose beads) was conducted. Indeed, the elevated GTP-bound Rac1 was found in cells following Rheb knock down, compared to cells treated with control siRNA (Figure. 3.2B.(i)). To overcome potential siRNA off-target effects, we employed three Rheb siRNAs targeting different regions of the Rheb mRNA. Consistently, depletion of Rheb expression with these siRNAs elevates Rac1 GTP levels (Figure.3.2B.(ii)), supporting our hypothesis that Rheb mediates Rac1 activation.

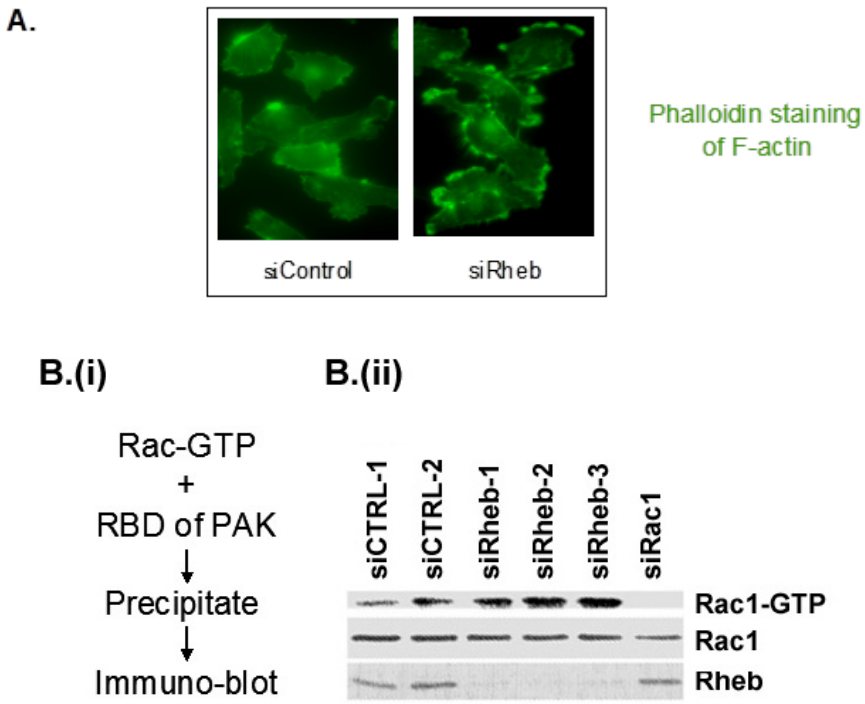

Figure 3.2 Knocking down Rheb expression in U373MG cells induces changes in the actin organization and Rac1 activation.

A. F-actin distribution. U373MG cells were transfected with 50 nM siRNA to Rheb and to control for 48 hours, followed by fixation and stained with fluorescently-tagged phalloidin.

B.(i). A protocol of Rac1-GTP pull-down assay; To measure Rac1-GTP level, three hundreds ug of cellular extract, from U373 MG cells previously transfected with siRNAs for 48 hours, were incubated with 20 µl of purified GST-PBD PAK1 at 4°C for 1 hour. The reaction mixtures were precipitated with glutathione-conjugated Sepharose beads, the amounts of GTP-bound Rac1 were determined by immunoblotting.

B.(ii). Three siRNAs to Rheb regulate Rac1-GTP levels. Rac1 siRNA was used as a negative control.

3.3 Suppression of Rheb expression induces cell migration

I next examined the biological relevance of Rheb-mediated Rac activation. chemotaxis assays were conducted to determine directional movement of cells depleted of Rheb toward chemoattractant, fetal bovine serum, as demonstrated in Figure 3.3A and 3.3B. Loss of Rheb accelerated U373MG cell migration across a permeable filter toward 0.1% FBS by ~2 fold, using a single Rheb siRNA as shown in Figure 3.3C; similar results were observed in cells treated with three unique Rheb siRNA s, compared to siRNA control, as shown in Figure 3.3D.

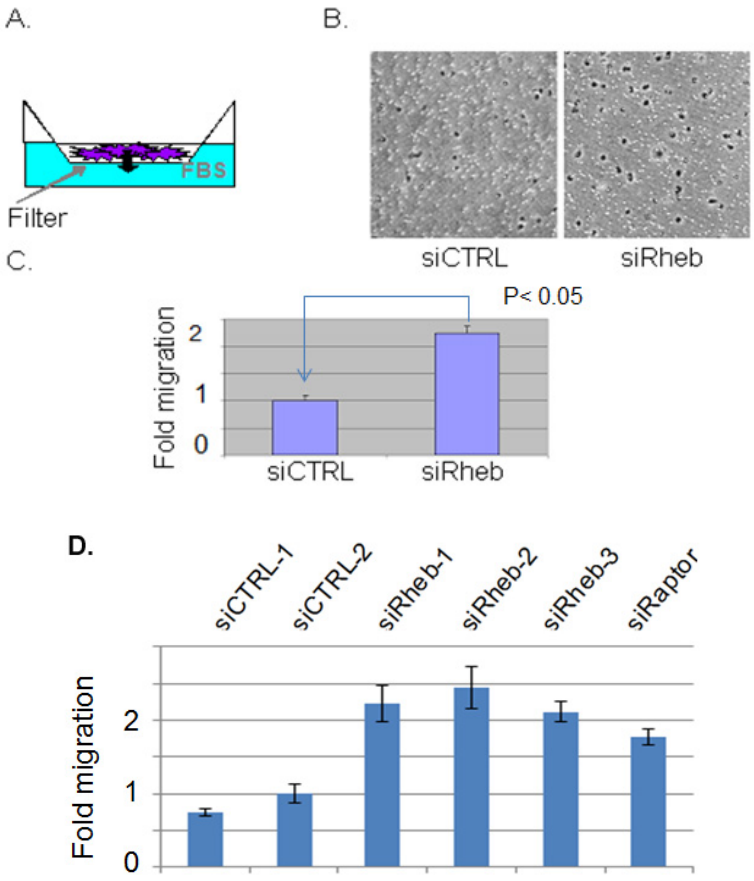

Figure 3.3 Suppression of Rheb affects cell migration.

A. Protocol of chemotaxis assay.

B. Following siRNA treatment for 48 hours, cells were loaded in the upper portion of the chamber, allowing cells to move through a permeable (8 μm pore size) filter toward 0.1% FBS present in the lower portion of the chamber. After 16 hours, cells that had migrated through the filter were fixed with 10% neutral buffered formalin, then stained with crystal violet dye and visualized by microscopy.

C. The bar graph shows averaged migration data from four experiments each using a single siRNA to Rheb. A student test program showed a statistical difference between siRNA Rheb and siRNA control, of $p < 0.05$.

D. Shown the rate of cell migration using three siRNA to Rheb, compared with control siRNA. Deletion of Raptor, a key mTORC1 component, served as a control.

3.4 Rheb-mediated S6 phosphorylation negatively regulates PDGF receptor expression

I next searched for the underlying mechanism of Rheb-mediated migration. Recently, it was reported that although the platelet-derived growth factor receptor (PDGFR) is an activator of PI3K but the receptor transcription is negatively regulated by mTORC1 [20], resulting in decreased receptor expression (Figure 3.4A). Since Rheb is upstream of mTOR in the PI3K pathway, we asked whether Rheb also affects PDGFR expression. We found that depleting Rheb mRNA increased PDGFR alpha expression in U373MG cells. Similar results were observed with two additional Rheb siRNAs. In addition, suppression of Raptor also elevated PDGFR expression, shown in Figure 3.4B. This result provided evidence that a negative feedback exists between mTOR signaling and PDGFR expression in U373MG.

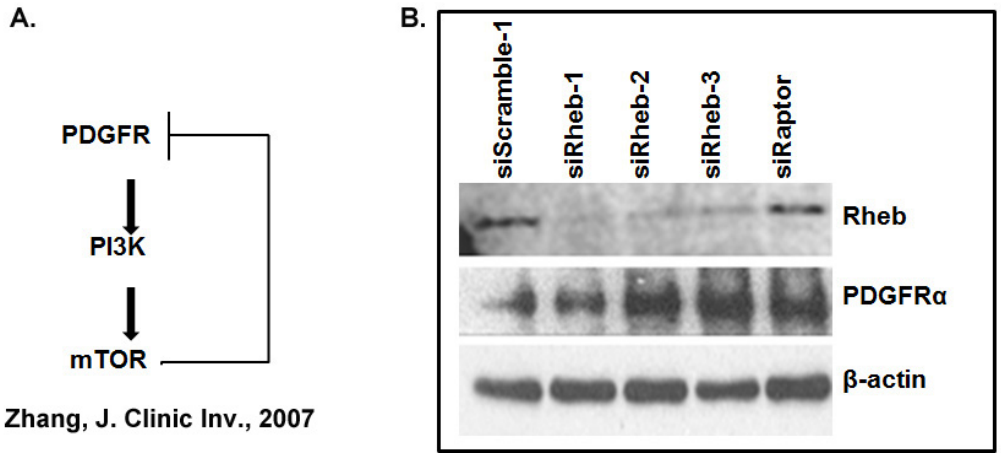

Figure 3.4 Knocking down Rheb increases PDGFR expression and PDFGR inhibitor, Gleevec, reduces Rheb-mediated migration.

A, A reported negative feedback loop between mTOR and PDGFR [20].

B. Knocking down of Rheb increases PDGFR expression. U373MG cells were transfected with 50 nM Rheb siRNAs and control siRNA. Forty eight hours thereafter, the cells were harvested. Aliquots of cell lysates containing equal amounts (20 ug) of proteins were analyzed by immunoblotting assay using anti-PDGFRα specific antibody, and ß-actin as a loading control.

3.5 PDGFR inhibitor, Gleevec, reduces Rheb-induced migration

PDGFR expression potentiates cell migration via PI3K/Rac pathway (36). To investigate whether Rheb-mediated PDGFR expression affects cell motility, I utilized Gleevec, a small molecule tyrosine kinase inhibitor to PDGFR, c-kit and Abl (37-38), and monitored the migration ability of cells following Rheb knock down. First, I determined Gleevec IC50 is ~10 μM (data not shown) to inhibit ERK phosphorylation, a read-out for PDGFR activation. Then, we conducted a migration assay using a range of doses of Gleevec. As expected, Rheb-induced migration was significantly repressed in the presence, compared to the absence, of Gleevec in a dose-dependent manner, demonstrated in Figure 3.5.

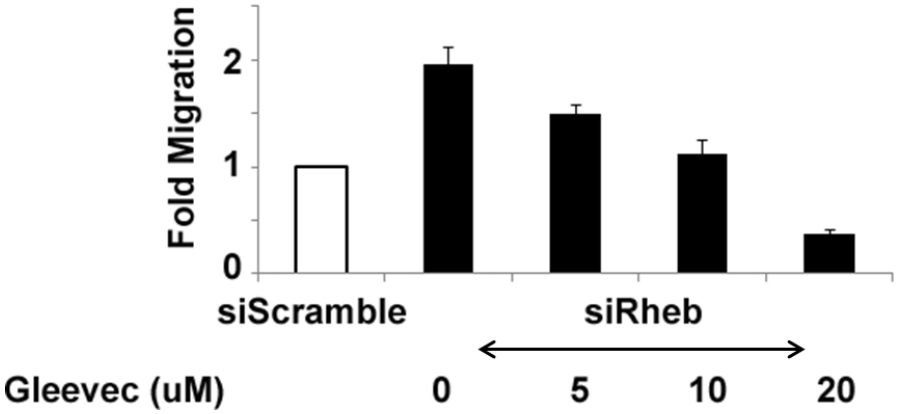

Figure 3.5 Gleevec, a PDGFR inhibitor, reduces Rheb-induced migration.

Following siRNA treatment for 48 hours, cells were pre-treated with Gleevec at 5,10 and 20 μM for 2 hours then cells were loaded in the upper portion of the chamber, allowing cells to move through a permeable (8 μm pore size) filter toward 0.1% FBS present in the lower portion of the chamber. After 16 hours, cells that had migrated through the filter were fixed with 10% neutral buffered formalin, then stained with crystal violet dye and visualized by microscopy. The bar graph shows averaged migration data from three experiments

3.6 Summary

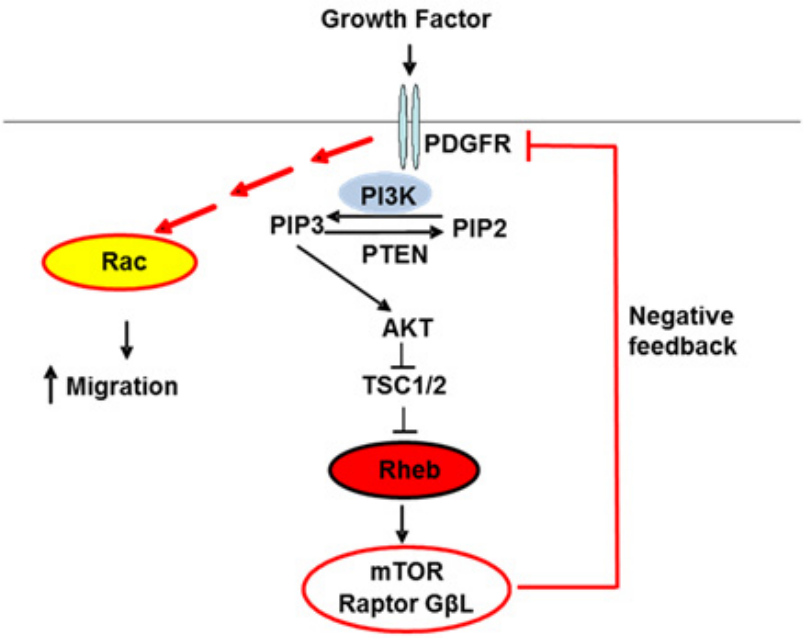

Figure 3.6 Model of Rheb/mTOR/PDGFR/Rac pathway in cell migration.

Rheb/mTORC1 signaling suppresses PDGFRα protein expression, which in turn diminishes Rac1-mediated actin polymerization and decreases cell migration.

3.7 Discussion

Cell migration is an important step for cancer metastasis; however the roles of Rheb1/mTORC1 in cell migration are not fully understood. There are controversial documents for Rheb's role in migration. Rheb increasing migration was reported in neuronal cells in newborn mice [139, 140]. Whereas others previously found that suppression of mTORC1 signaling inhibits tumor growth but does not prevent cancer progression in a thyroid cancer model [141]. It may be due to the different genetic backgrounds of the tested cell lines. Our lab was interested in a potential role of Rheb, in glioblastoma thus U373MG, a PTEN-deficient human glioblastoma cell line, was selected in this study. We found that knocking down Rheb induces F-actin reorganization and Rac1 activation, consequently enhancing cell migration in the U373MG cells. Furthermore, abrogation of Raptor, a Rheb downstream mTORC1 component had similar results - increasing migration, confirming that Rheb/mTOR decreases the migration of glioma cells.

I found that the mechanism underlying this action is that Rheb knockdown induces a negative feedback loop that activates PDGFR expression and Rac1 activation. In addition, depletion of Raptor behaves similarly. Zhang, et al. (2007) reported that in TSC1/2-/- (upstream negative regulator of Rheb) cells, Akt activation is remarkably reduced in response to serum and PDGF stimulation, along with a reduction in cell ruffling. PDGFRα expression is tremendously decreased in this cell line [20]. This illustrates an mTOR negative feedback loop on PDGFR that is consistent with our findings. mTOR exists in two different

complexes, mTORC1 and mTORC2. It is possible that there is crosstalk between these two complexes. For example, inhibition of mTORC1 by DEPTOR overexpression activated mTORC2 by releasing mTORC1's negative feedback loop on mTORC2 [142]. There are also reports that inhibiting mTORC1 with rapamycin lead to mTORC2 activation in multiple cancer cell lines [23]. Thus, it would be interesting in future to measure mTORC2 substrate Akt Ser473 phosphorylation in this study.

PDGF is an activator of Rac1 and stimulates Rac1-dependent migration of fibroblasts and vascular smooth cells [143, 144]. We found that treating Rheb-depleted cells with PDGFR inhibitor Gleevec reverses Rheb-depletion-induced cell migration, suggesting that PDGFR is in the Rheb/mTOR/Rac1 pathway. Since this small molecular may also target other proteins in the cells, it would be interesting to have additional approach such as knockdown of PDGFR to further confirm the mechanism of this action.

In term of clinical implication, my works suggest that when treating cancer with Rheb/mTOR inhibitors, optimal chemotherapy may be achieved by addition of a migration inhibitor, such as PDGFR inhibitor, to reduce both cell growth and cell migration/metastasis.

CHAPTER 4 RESULTS

mTOR REGULATES AURORA A VIA ENHANCING PROTEIN STABILITY

4.1 mTORC1 is active during G2/mitosis in mammalian cells.

The function of mTOR in the G1 phase of the cell cycle has been extensively studied, while its role in G2 and mitosis is much less understood. To explore the role of mTORC1 signaling on mitosis, I first examined whether there was a correlation between mTOR activity and G2/M phase progression. HeLa, human cervical cancer cells, were utilized. These cells were selected because (1) they were derived from a cancer, (2) they are a commonly used human cell line in many research fields, such as infectious disease [145], AIDS [146],and cancer [147]. (3) Most importantly, HeLa cells can be effectively synchronized in S phase entry by removal of thymidine. Release of the cells from thymidine blockade enabled us to follow gene expression and other events when the cells subsequently pass through the cell cycle. As shown in Figure 4.2.1, HeLa cells were accumulated in the G1/S phase by double thymidine blockage/release, measured by FACS analysis. At 3.5, 4 and 5 hours post release, cells entered the early, middle and late S phases, respectively, and at 8 hours were in the G2 phase, shown by 4N DNA and cyclin B1 reaching its peak expression. At 10 hours post release, cells entered the mitotic phase, demonstrated by the mitotic marker histone H3 phosphorylation peak. To monitor mTOR activity, phosphorylation of its downstream substrates, S6K1 and 4E-BP1, was measured at various time points of the cell cycle by quantitative immunoblotting analysis. As shown in Figure 4.1, when cells entered the S phase, phosphorylation of S6K1 started to increase and reached its peak during the G2/M phases; 4E-BP1 phosphorylation, including theT37/46 and S65 sites, was also elevated in the

G2/M phases, notably S65 was remarkably increased. These results indicated that mTOR activity preceded or coincided with the G2/M phases and suggest that mTOR may be involved in regulation of mitosis during the cell cycle.

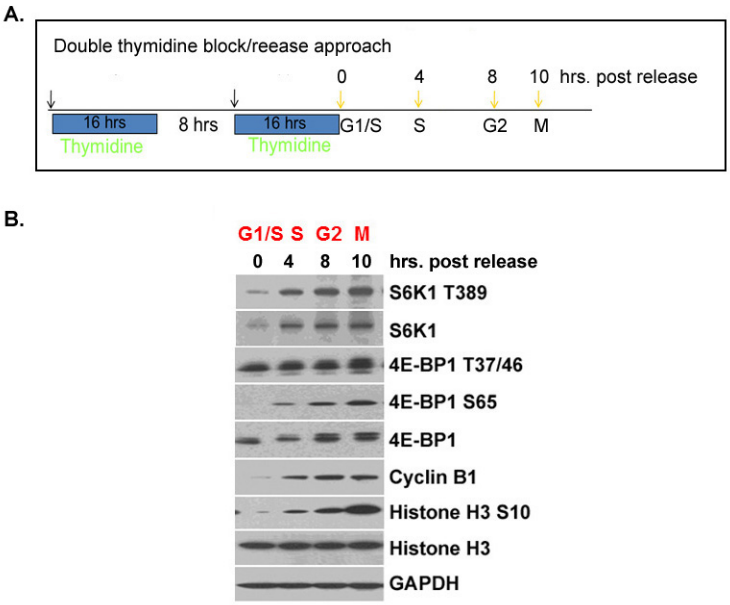

Figure 4.1 mTOR signaling is higher in G2/M phases

A. Schematic of double thymidine block/release assay.

B. HeLa cells were synchronized with double thymidine block/release, as indicated in Methods and Materials. At 4 hours cells reached S phase, 8 hours G2, and 10 hours mitosis. The cellular proteins were collected and the expression levels and phosphorylation status of mTOR substrates, S6K1 and 4E-BP1, were determined by immunoblotting assay using antibodies specific against phospho- and total proteins.

4.2 Inhibiting mTOR signaling suppresses mitotic progression

To address whether mTOR affects mitotic progression, I utilized the well-characterized mTOR inhibitor, rapamycin to inhibit mTOR activity. Subsequently, we monitored mTOR effects on cell cycle progression by immunoblotting for the mitotic marker phospho-Histone H3 Ser10 (S10) and monitored cell population change by flow cytometry. Rapamycin was administered at 4 hours or 5 hours post thymidine release based on the flow cytometry (Figure.4.2), to avoid drug interference with G1- or S-phase progression. The results showed that rapamycin treatment abolished S6K1 T389 phosphorylation. Interestingly, 4N DNA containing cells were reduced by 10% and mitotic maker Histone H3 S10 was also decreased in the M phase, compared to vehicle control. These findings support the above notion that mTOR regulates the mitosis phase of the cell cycle.

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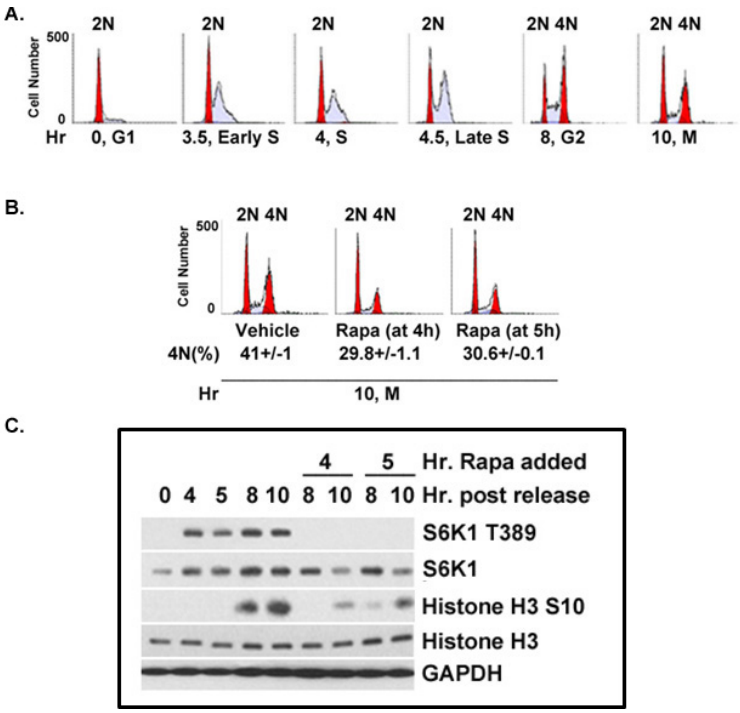

Figure 4.2 Inhibiting mTOR signaling suppresses mitotic progression.

A. Flow cytometry shows that at 3.5 and 5 hours post release from thymidine block, cells were in the course of the S phase. It suggests that administering mTOR inhibitor at 4 or 5 hours post release should not interfere with S phase progression.

B. HeLa cells were treated with rapamycin at 4 and 5 hours post release from thymidine, and at 10 hours the cell populations were analyzed by FACS. The data are representative of two experiments.

C. Cellular lysates were collected from duplicated samples and mTOR substrate S6K1 and mitotic marker Histone H3 S10 were examined by immunoblotting assay using antibodies specific against phosphor- and total proteins.

4.3 Comparison of using double thymidine to RO-3306 in synchronizing cells

Use of double thymidine block (via inhibition of DNA synthesis) to synchronize cells is very time-consuming [148, 149]. It takes about 40 hours to synchronize cells at G1/S phase, including a 1st16 hours thymidine-treatment to block; wash/release cells into the cell cycle for 8 hours; then a 2nd thymidine treatment for 16 hours, followed by wash and release to allow cells to enter the cell cycle. In addition, it takes about an additional 8 hours from the G1/S to G2/M phases, in which we are interested, see Figure 4.2. Thus, when reaching to G2/M, cell populations may already be regaining an asynchronous state [149].

Recently, Vassilev, et al proposed a simple, fast method to synchronize cells by inhibition of Cdk1activity, based on well-known, essential role of Cdk1/cyclinB in G2/M transition [148] In this method, a selective, reversible Cdk1 inhibitor, RO-3306, is used for treating cells to block proliferating cells at the G2/M phase transition site. After washing off the drug, the cells are released from the inhibitory effect and rapidly enter M phase. In this one single‑step procedure for about 20 hours, one can generate highly synchronous populations of G2/M cells which are useful for both cytological and biochemical studies [95, 149]. A comparison of thymidine block to RO-3306 in synchronizing cells is showed in Figure 4.3. Therefore, I utilized RO-3306 in my subsequent studies for synchronization of HeLa cells.

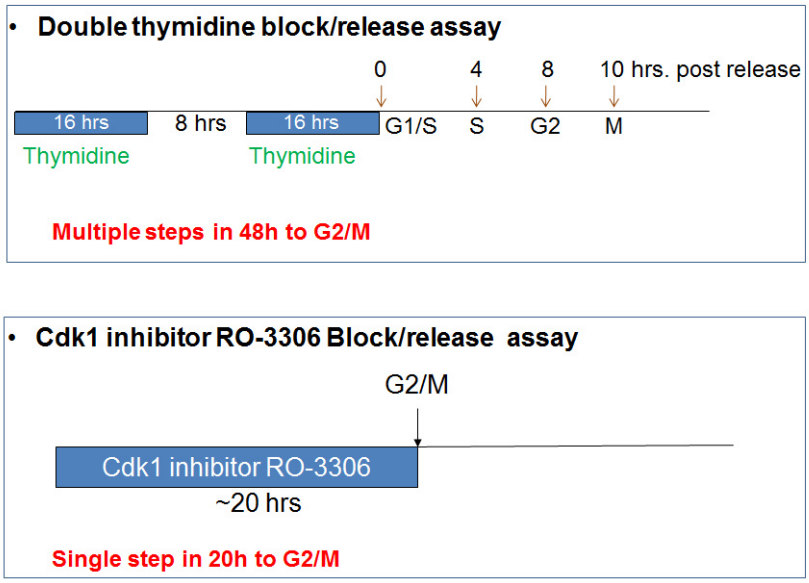

Figure 4.3 Comparison of using double thymidine to RO-3306 in synchronizing cells

A. A double thymidine block/release approach takes multiple steps over a 48 hours time-period to reach a synchronized G2/M transition.

B. Treatment with Cdk1 inhibitor RO-3306 only takes a single 20 hours treatment to block cells at G2/M border.

4.4 Inhibiting mTOR signaling suppresses mitotic progression, continued

As see in Figure 4.4, 72 % of cells had 4N DNA contents, which were analyzed by fluorescence-activated cell sorting (FACS). The phosphorylation of mitotic maker Histone H3 S10 was faint when analyzed by immunoblotting, indicating that cells were in the G2 phase.

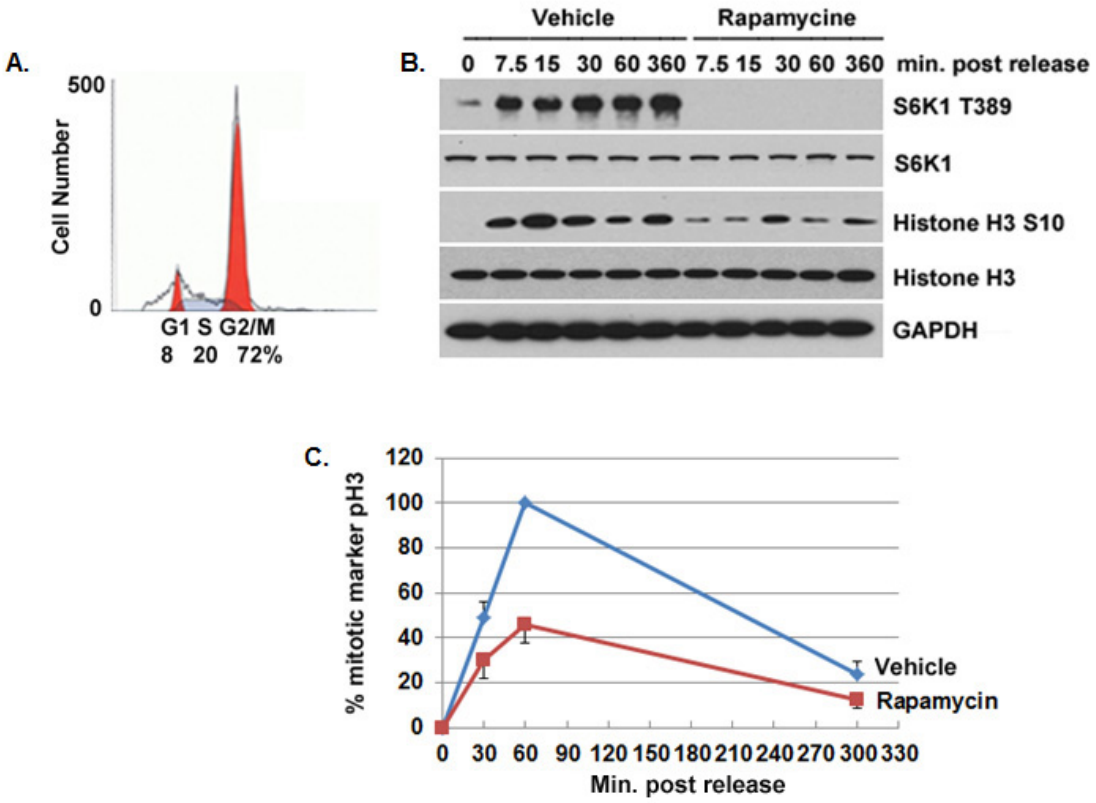

Figure 4.4 Inhibiting mTOR signaling suppresses mitotic progression.

HeLa cells were synchronized with Cdk1 inhibitor RO-3306 for 19 hours.

A. The synchronization efficiency was determined by FACS as described previously.

B. The cells were then treated with mTOR inhibitor rapamycin 100 nM for 2 hours, followed by washing and releasing cells into the cell cycle. One set of the samples were continued to treat with mTOR inhibitor by adding rapamycin immediately after release and another set with vehicle DMSO. The cellular

extracts were harvested at indicated times, and mitotic marker Histone H3 S10 and mTORC1 substrate S6K1 were examined by immunoblotting assays.

C. Diagram showing quantitation of Histone H3 S10 phosphorylation from four experiments.

4.5 Aurora A mitotic kinase is most obviously affected by mTOR

Mitosis progression is tightly regulated by multiple protein kinases, such as ataxia telangiectasia mutated (ATM), check 1 (Chk1), Aurora A, and polo-like kinase 1 (Plk1)**,** and cyclin-dependent kinase 1 (Cdk1) to ensure DNA integrity and chromosome being aligned properly before DNA segregation and cell division. Based on these well-documented facts, I asked which mitotic kinase is regulated by mTOR signaling. HeLa cells were treated with mTOR inhibitor rapamycin for 2 hours and immunoblotting assays were shown in Figure 4.5.1, Aurora A protein level and its phosphorylation on T288, an active site on its T loop, were obviously down regulated, while .ATM, Chk1, and Plk1 and Cdk1 proteins remained unchanged. Interestingly, cells treated with the mTOR inhibitor displays monopolar chromosomes, which is one of characters of Aurora A defects due to centrosome duplication and spindle assembly (Figure 4.5.2). These results suggest that Aurora A might be a target for mTOR regulation. Thus, I selected Aurora A in this study to explore how mTOR regulates Aurora A mitotic protein.

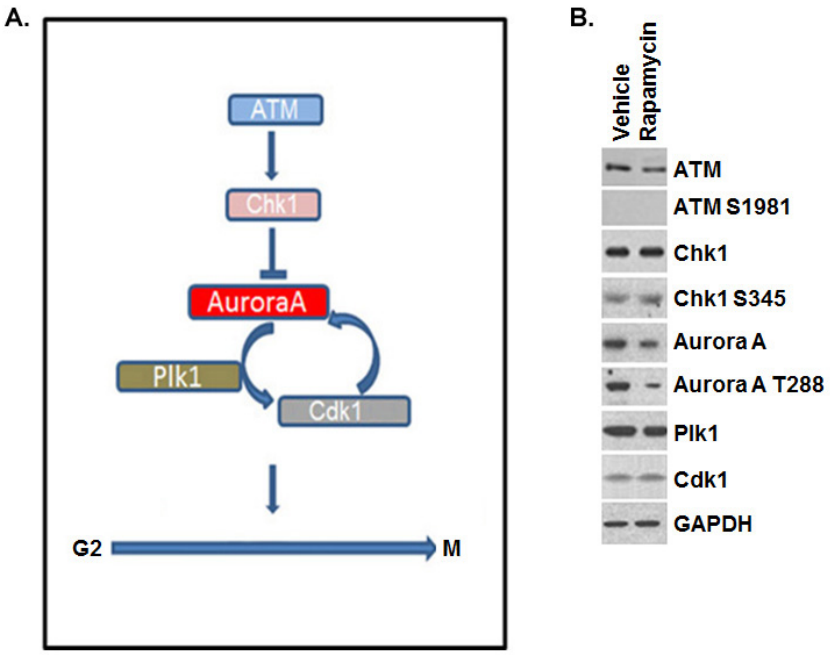

Figure 4.5.1 Aurora A is most obviously affected by mTOR inhibition.

A. Mitotic kinase signaling from ATM, to Chk1; Aurora A, Plk1; and Cdk1.

B. HeLa cells were treated with mTOR inhibitor rapamycin 100 nM for 2 hours. The cellular lysates were collected and equal proteins were subject to SDS-PAGE. The kinases described above were examined by immunoblotting assays using specific antibodies against ATM, Chk1, Aurora A, Plk1 and Cdk1 proteins.

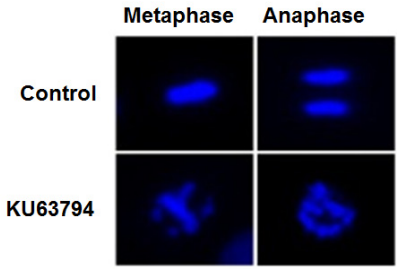

Figure 4.5.2 Misalignment of chromosomes and monopolar chromosomes were observed in HeLa cells.

HeLa cells were treated with mTOR inhibitor KU63794 1μM for 24 hours, then fixed with 4% paraformaldehyde. The chromosomes were stained with DAPI and visualized by fluorescence microscopy.

4.6 Blocking mTOR activity reduces Aurora A protein levels

Aurora A is a key Ser/Thr kinase protein that plays important roles not only in mitosis of the cell cycle, but also in tumorigenesis. Thus Aurora A became a prime candidate for further testing. In this study, two types of synchronization approaches: double thymidine blockage method and Cdk1 inhibitor RO-3306 blockage/release were utilized. At both experiments, mTOR inhibitors reduced Aurora A protein levels shown in Figure 4.6.1 and 4.6.2. These results suggest that mTOR regulates Aurora A expression, at least at the protein level. Further, phosphorylation of histone H3 S10, which is spatio-temporal regulated by Aurora A kinase [150], was diminished after rapamycin and Torin1 treatment during mitotic progression. These findings support the hypothesis that mTOR regulates mitotic Aurora A during the cell cycle.

Another phenomenon observed is that rapamycin (an allosteric inhibitor) and Torin1 (a kinase catalytic inhibitor) have different effectiveness of inhibiting phosphorylation of S6K1 vs. 4E-BP1, the two substrates of mTORC1, see Figure 4.6.1, both mTOR inhibitors abolished phosphorylation of the S6K1, and Torin1 also completely suppressed phosphorylation of 4E-BP1, including its sites at T37/46, S65, and T70 but rapamycin only partially affected phosphorylation status of 4B-BP1. These data indicate that the allosteric effect is not sufficient to completely inhibit mTORC1 activity, while the kinase active site inhibitor does. This agreed with others reports [57, 60, 151]. Based on these observations, Torin1 and another ATP-competitive inhibitor, KU63794 were used in my subsequent experiments.

A.

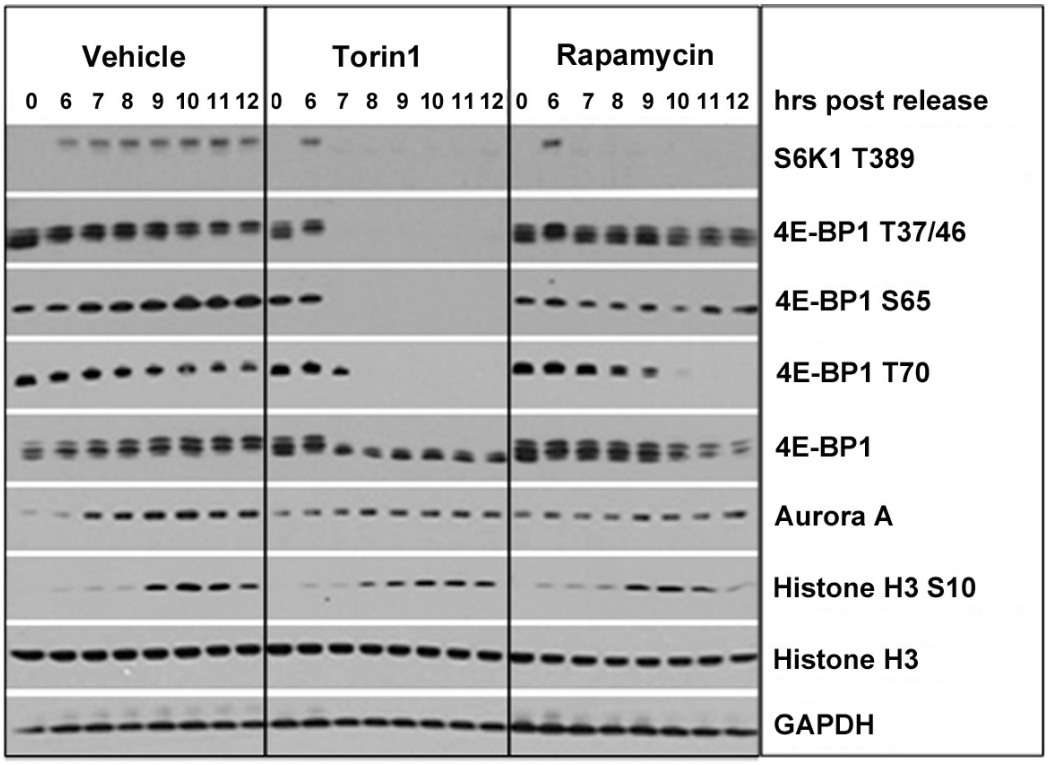

Figure 4.6.1 Blocking mTOR activity reduces Aurora A protein level.

HeLa cells were synchronized by double thymidine blockage/release. At 6 hours post release, mTOR kinase catalytic inhibitor Torin1 250 nM or mTOR allosteric inhibitor rapamycin 100 nM were administered. Cellular lysates were collected at the indicated times post release and equal amounts of proteins were subject to immunoblotting assay with specific antibodies as indicated.

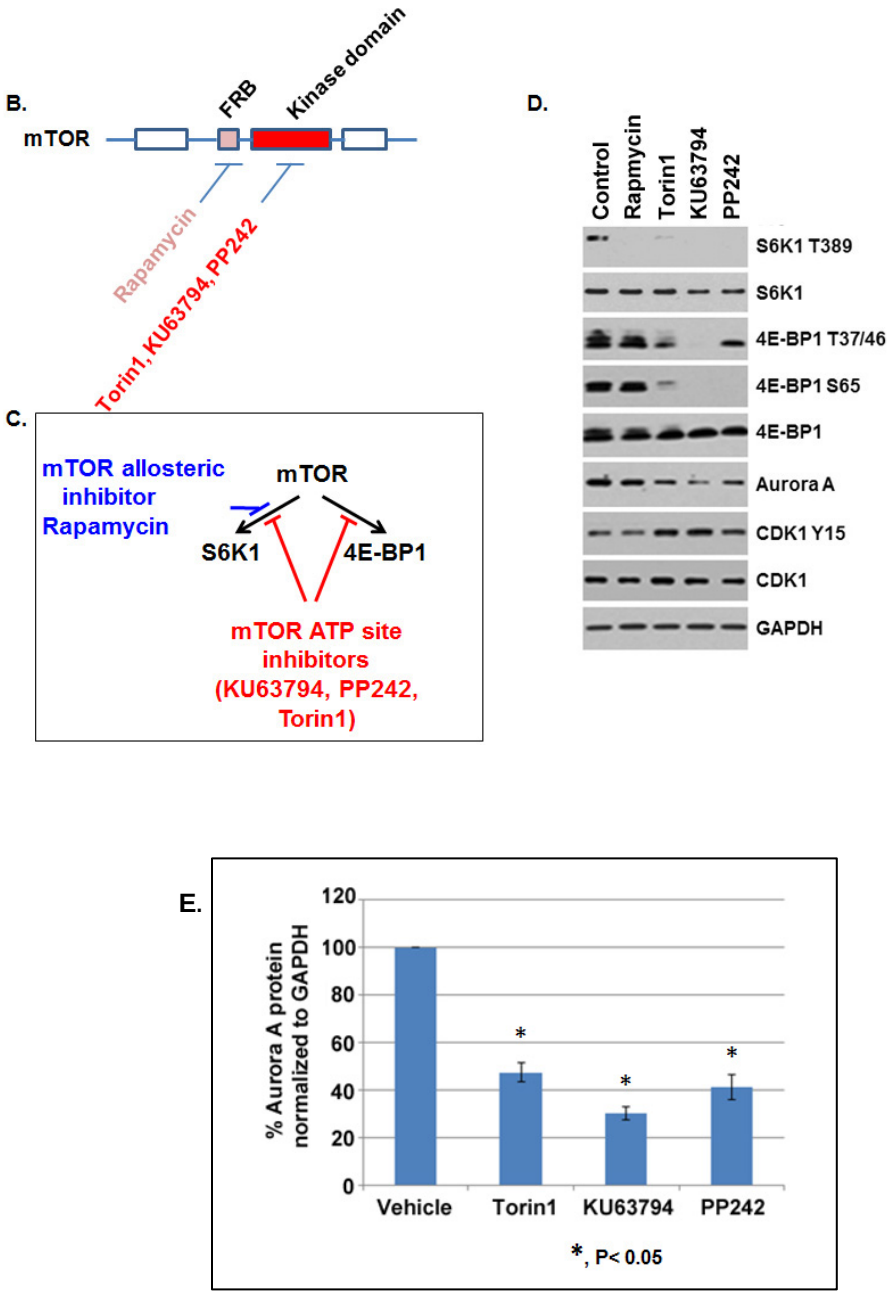

Figure 4.6.2 Blocking mTOR activity reduces Aurora-A protein level, continued.

B. Diagram indicating mTOR protein structure, kinase domain and FKBP12 binding domain

C. Diagram showing mTORC1 has two well-known substrates, S6K1 and 4E-BP1. Rapamycin binds FKBP12, then this complex bind the FKBP12-Rapamycin

Binding (FRB) domain, an allosteric site of mTOR, inhibiting indirectly mTORC1 activity. Torin1, KU37694, and PP242 directly bind and repress mTOR kinase activities. They are ATP-competitive mTOR kinase inhibitors that block all mTORC1 and C2 activity while rapamycin preferentially inhibits mTORC1-mediated phosphorylation of S6K [151].

D. HeLa cells were incubated with RO-3306 5uM for 19 hours then pre-treated with rapamycin, Torin1, KU63794, or PP242 for 1 hour (still in presence of RO-3306). The cells then were washed with warm medium and released to the fresh medium, followed by immediately treating with indicated inhibitors or DMSO. Cells were lysed at 2 hours after release. Equal amount of cellular proteins were subject to immunoblotting assays using specific antibodies against S6K1, 4E-BP1, Aurora A, and CDK1proteins, GAPDH was used as a loading control.

E. Quantitation of Aurora A proteins. Results represent the means ± the standard errors from three experiments. * indicates $p < 0.05$ compared to vehicle control using a two-tailed, paired Student's t test.

4.7 Potential mechanisms for mTOR regulation of Aurora A

It is known that mTOR controls certain genes of transcription [20] or translation [79], and/ or protein stability [79]. However, it is unknown whether mTOR regulates Aurora A through one or more these ways, I attempted to address these questions one by one as follows.

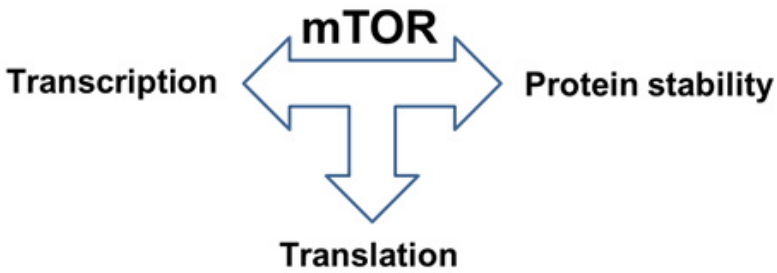

Figure 4.7 Putative approaches for mTOR regulation of Aurora A.

4.8 mTOR does not affect Aurora A transcription

The detected Aurora A protein level change could have been the result of altered gene transcription, transcript stability, translation and/or protein stability. Therefore, I examined whether mTOR mediates AURKA transcript levels. Since AURKA transcription mostly occurs in the G2 phase of the cell cycle [152], HeLa cells were blocked at the G2/M border utilizing Cdk1 reversible inhibitor RO-3306 [148], followed by treatment with mTOR allosteric inhibitor rapamycin and active-site direct inhibitors TORin1, KU63794 [61], and PP242. TaqMan quantitative PCRs were carried out to analyze the mRNA levels of AURKA using AURKA specific primer/probe. As shown in Figure 4.8, the mRNA messages remained unchanged in rapamycin treated cells as well as Torin1, KU63794, and PP242 treated samples, indicating that mTOR has minimal effect on the steady-state AURKA transcription levels.

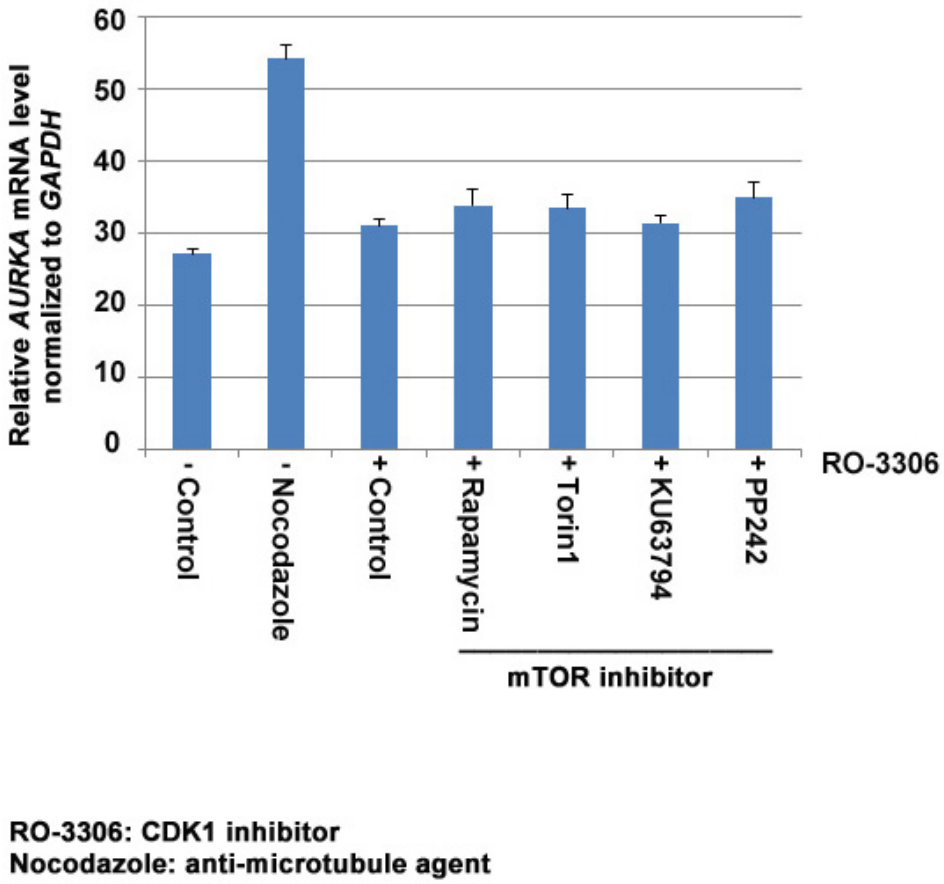

Figure 4.8 mTOR does not affect Aurora A transcription

HeLa cells were incubated with RO-3306 5μM for 15 hours, then treated with mTOR inhibitors rapamycin 100nM, Torin1 250nM, KU63794 1μM, and PP242 750nM for additional 4 hours. For a mitotic control, cells were treated with Nocodazole 100nM for 19 hours. Total RNAs were isolated and quantitative PCRs were conducted using specific primer/probe against AURKA and GAPDH mRNAs. Nocodazole is an anti-microtubule agent, which interferes with the polymerization of microtubules and dynamic microtubule network to form the mitotic spindle. Thus Nocodazoleblocks cells at mitosis, served as a control. The data represent three independent experiments.

4.9 Suppression of mTOR affects translational apparatus assembly in HeLa cells

I next asked whether mTOR regulates Aurora-A at the translation level. To examine this possibility, we first performed a cap-binding experiment to see if mTOR inhibitors affect translation apparatus assembly in this cell line. I treated cells with various mTOR inhibitors for 2 hours to block canonical mTORC1-dependent events and capture the cellular 5' cap complex with m^7GTP-Sepharose beads that mimic the mRNA 5' cap structure. The results (Figure 4.9) showed that 4E-BP1 was largely retained on m^7GTP-Sepharose following treatment with mTOR inhibitors rapamycin, Torin1, KU63794 or PP242, coincident with reduced association of eIF-4G1 with eIF4E. These effects were more obviously seen using inhibitors targeting the kinase domain versus rapamycin, suggesting that potential mRNA translational regulation may primarily be regulated by 4E-BP1, in agreement with other investigators [69, 153].

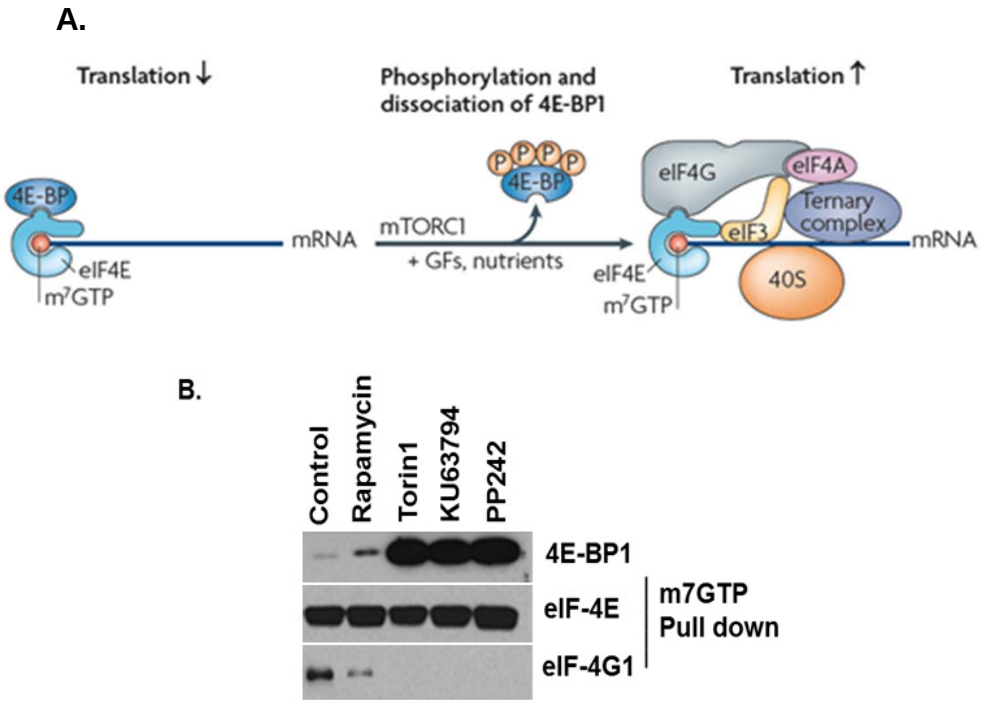

Figure 4.9 4E-BP1 competes off eIF-4G1 binding to eIF4E with mTOR inhibitors.

A. Model of translational apparatus assembly. In the absence of nutrients or GF, non-phosphorylated 4E-BP1 binds tightly to the translation initiation factor eIF4E, preventing it from binding to 5'-capped mRNAs and recruiting them to the ribosomal initiation complex. In the presence of nutrients or GF, mTOR phosphorylates 4E-BP1, resulting in liberation of eIF4E and its recruitment into the translation-initiation-factor complex (eIF4F), which includes eIF4A and eIF4G, so promoting mRNA translation. The figure was obtained from [154].

B. **I**nhibition of mTOR affected translational apparatus assembly in HeLa cells. Cells treated with the indicated mTOR inhibitors for 2 hours were subject to m^7GTP cap binding assay. Briefly, cell extracts were incubated with m^7GTP-Sepharose beads (that mimic the mRNA 5' cap structure), then washed and bound proteins resolved by SDS-PAGE. 4E-BP1, eIF-4G, and eIF4E proteins were examined using specific antibodies in immunoblotting assays.

4.10 mTOR facilitates Aurora A translation via short transcriptional splicing

Based on the above data for the global effect of mTOR-mediated translation, I next examined whether mTOR mediates specific gene AURKA mRNA translation. Since there are 6 splice variants for the AURKA in mammalian cells, each having a different 5'UTR (Figure 1.6) but sharing a common coding region, I first asked which transcript variant(s) exist in HeLa cells, the cell line that has been used throughout this study. To address this question, I carried out a 5' RACE assay to isolate 5' ends of AURKA from HeLa (Figure 4.10.1). Sequencing revealed that among nine isolated clones, eight were variant 5 isoform of AURKA 5' UTR and one was an unrelated sequence. The transcriptional start site was determined as the first nucleotide that is 3' to the adapter sequence ligated to the 5'of the mRNA transcripts. Additionally, using specific primers for each alternatively splicing variant of AURKA un-translated region, the variant 6 of the gene was also found in 5' RACE mixture extracted from the HeLa cells, albeit at much lower abundance than variant 5, in Figure 4.10.2. Thus, two isoforms of 5' leaders, variant 5 and 6, were identified in the HeLa cells. Variant 5 is 243 nucleotides in length, containing the exons I and II, whereas the latter is 133 nucleotides, containing only exon I. Interestingly, AURKA variant 5, but not 6, has a single upstream ORF (uORF) encoding 39 residues. This uORF is out of frame with the initiating ATG in the 5' UTR AURKA and terminates after 39 codons at a TGA within the coding region of AURKA (Figure 4.10.3). I anticipated that this uORF might potentially force ribosomes to prematurely initiate translation at the

uORF and pass the true initiating ATG and drop off without translating Aurora A as has previously been described for CHOP [133]. These features might confer translational regulation of Aurora A by the mTOR/eIF4E pathway. To address whether the 5'UTR sequence of the AURKA transcript plays a role in its translation control, I used a pTK-Aurora A-Luc reporter, which contained a cDNA segment encoding the human AURKA 5'UTR segments of variant 5 or 6 fused to firefly luciferase [133] downstream of the minimal TK promoter in PGL3 plasmid. These constructs were transfected into HeLa cells, and luciferase activity measured following treatment of cells with mTOR inhibitor KU63794 for 2 hours. Surprisingly, the Luc activity derived from the variant 5 reporter plasmid was not significantly affected by mTOR inhibition with KU63794. In contrast, the luciferase activity derived from the shorter variant 6-containing reporter was decreased 4 folds upon mTOR inhibition (Figure 4.10.4). These findings suggested that a short rather than a long, structurally complex 5'UTR might be most responsive to mTOR regulation. This contradicts earlier studies [95] but is consistent with a recent observation by Thoreen et al. who found that short 5'-primidine-rich sequences were most responsive to inhibition by Torin1 [69]. This will be discussed in detail later.

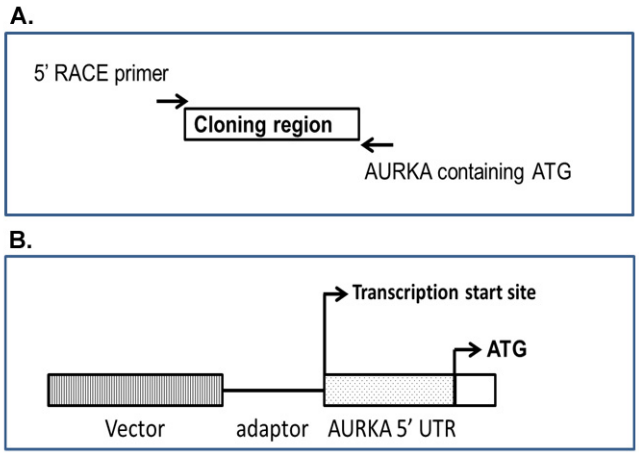

Figure 4.10.1 Alternatively spliced AURKA variant 5 found in HeLa cells.

Cloning of 5'UTR AUKRA from HeLa extract using 5' rapid amplification of cDNA ends (5'RACE)

A. For cloning specific 5' UTR in HeLa, 5' primer containing an adaptor sequence to capture 5' cap mRNA; 3' primer covering 5' end of Aurora A coding region were used for RT-PCR and resulting fragments were inserted into a TA cloning vector for sequencing.

B. Sequencing revealed that among nine clones, eight were variant 5 isoform of AURKA, and one sequence was non-specific. The transcriptional start site was determined as the first nucleotide that is 3' to the adapter sequence ligated to the 5'of the mRNA transcripts.

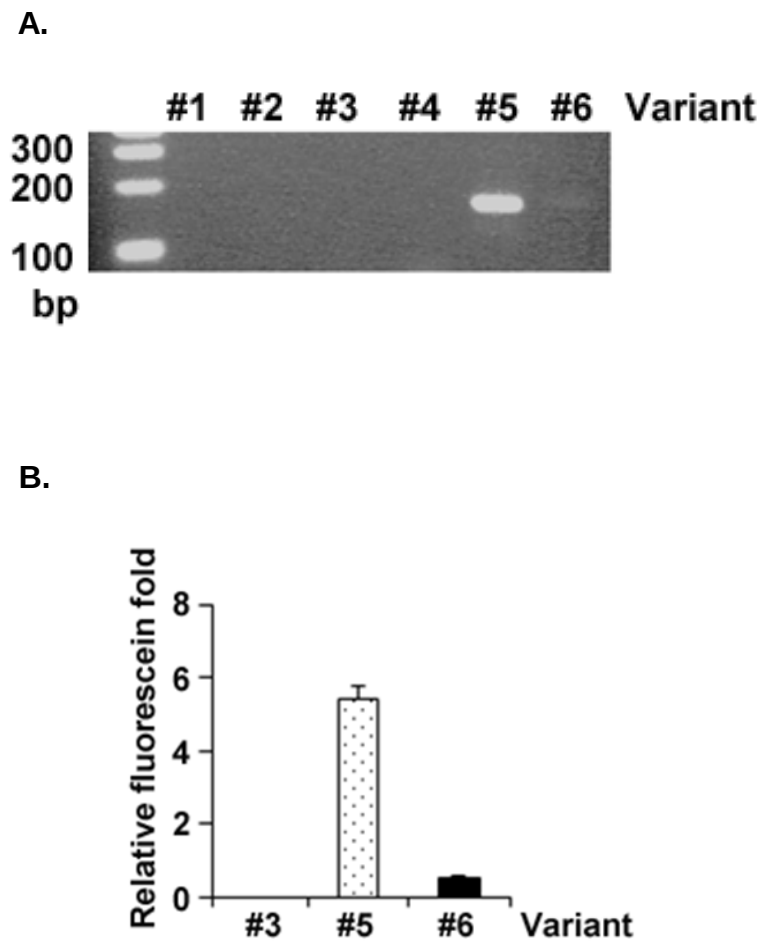

Figure 4.10.2 Aurora A transcript splicing variant 6 exists in HeLa cells.

A. PCR showing amplification of variants 5 and 6 (albeit faintly) in HeLa. Six reverse primers, corresponding to six distinguishing variants and F1 forward primer (see APPENDIX 1), which is located in the adjacent translation start site, were used in this experiment. The PCR products were separated in a 4% agarose and visualized by staining of ethidium bromide.

B.SYBR, a cyanine dye that binds to DNA, was used for quantification of the variants # 3, #5 and #6. The primers for SYBR were identical for the PCR described above.

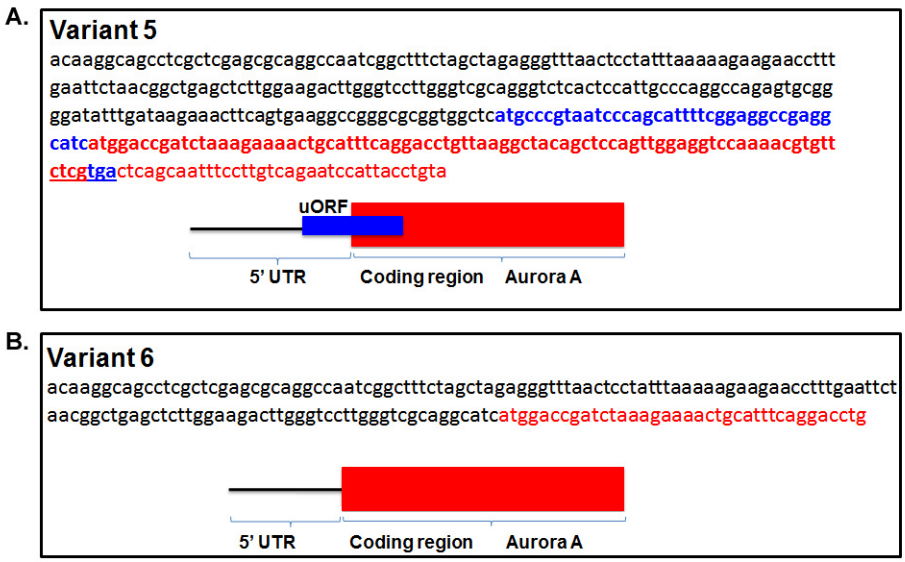

Figure 4.10.3 Aurora A variant 5, but not 6, has a single upstream ORF

A. Alternative splicing variant 5. A uORF in variant 5 is out of frame with the initiating ATG in the 5' UTR Aurora A and terminates after 39 codons at a TGA within the coding region of Aurora A. AURKA coding sequence is shown in red. The portion of the uORF prior to the true ATG, along with the uORF stop codon is shown in blue. Cartoon is not drawn to scale.

B. Alternative splicing variant 6. This variant contains only a short 5'UTR with no uORF.

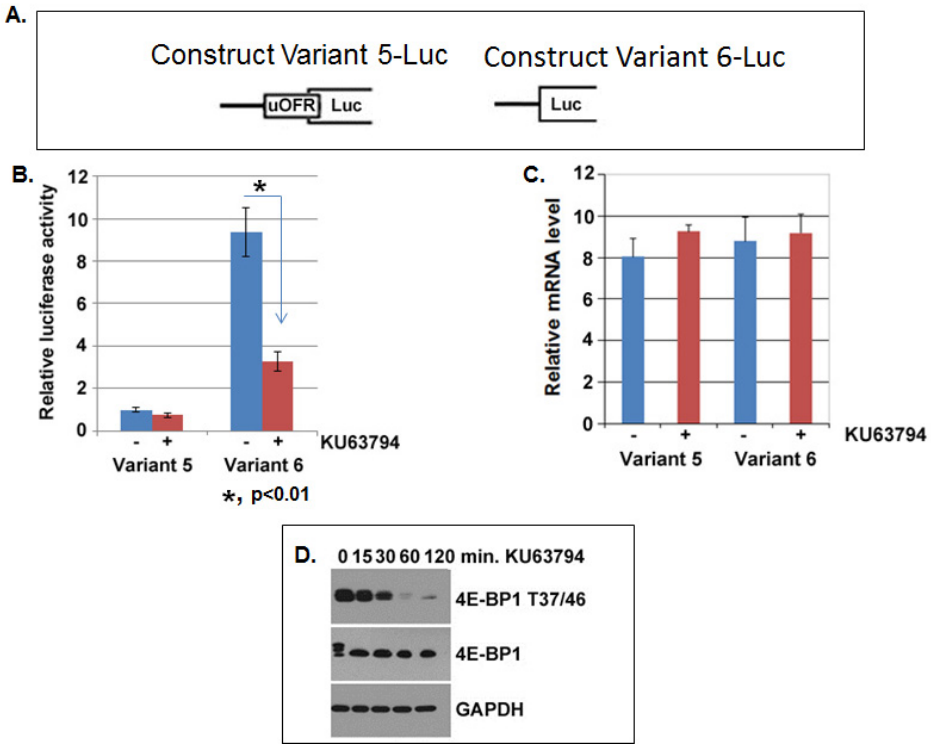

Figure 4.10.4 Short 5' UTR is sensitive to mTOR inhibition.

A. Constructs of Aurora A variant 5 (or 6)-Luc reporters. The Variant 5 contains exon I and II while the Variant 6 only exon I. A stop codon inframe with the uORF but out of frame with luciferase was naturally present within the luciferase sequence.

B. Luciferase assays were performed in HeLa cells that had been transfected with the illustrated constructs for 24 hours, followed by treatment with mTOR inhibitor KU63794 for 2 hours. Results represent the means ± the standard errors from three experiments. * indicates $p < 0.01$ compared to vehicle control using a two-tailed, paired Student's t test.

C. The levels of Luciferase mRNAs in duplicated samples were measured by RT-PCR using primer/probe that specifically recognized luciferase mRNA, and normalized to cellular GAPDH mRNA.

D. The phosphorylation levels of mTOR substrate 4E-BP1 T37/46 at indicated time were examined by immunoblotting assays, using antibodies specific against 4E-BP1 and its T37/46. GAPDH protein was used as a loading control.

Thus, my data is agreement with the Thoreen/Sabatini's results [69] than the EGFR/exon2 study. That is, a 5'-primidine-rich sequence rather than a complex 5'UTR is more responsive to mTOR activity.

4.11 Blocking mTOR accelerates Aurora A protein destruction

The above findings suggest minimal regulation of mRNA transcription/stability and only a minor AURKA transcript is responsive to mTOR inhibition. Besides regulating transcription and translation, mTOR reportedly can maintain the stability of proteins such as cyclin D [79]. If mTOR maintains Aurora A protein stability, inhibiting mTOR would accelerate Aurora A destruction. HeLa cells were pre-treated with protein synthesis inhibitor cycloheximide (CHX) 50 ug/ml [155] for 30 minutes before being exposed to mTOR inhibitor KU63794 for 4 hours. Cells were harvested at different times after KU63794 treatment (t=0) and protein levels were analyzed by quantitative immunoblotting assays. As seen in Figure 4.11, Aurora A was rapidly degraded in the presence of CHX. This occurred much more rapidly in cells co-treated with mTOR inhibitor KU63794. Within 30 minutes, the total amount of Aurora A started to decrease in the mTOR-inhibited cells. For control cells, it similar as reported previously [99]. This suggests that mTOR might prevent Aurora A degradation.

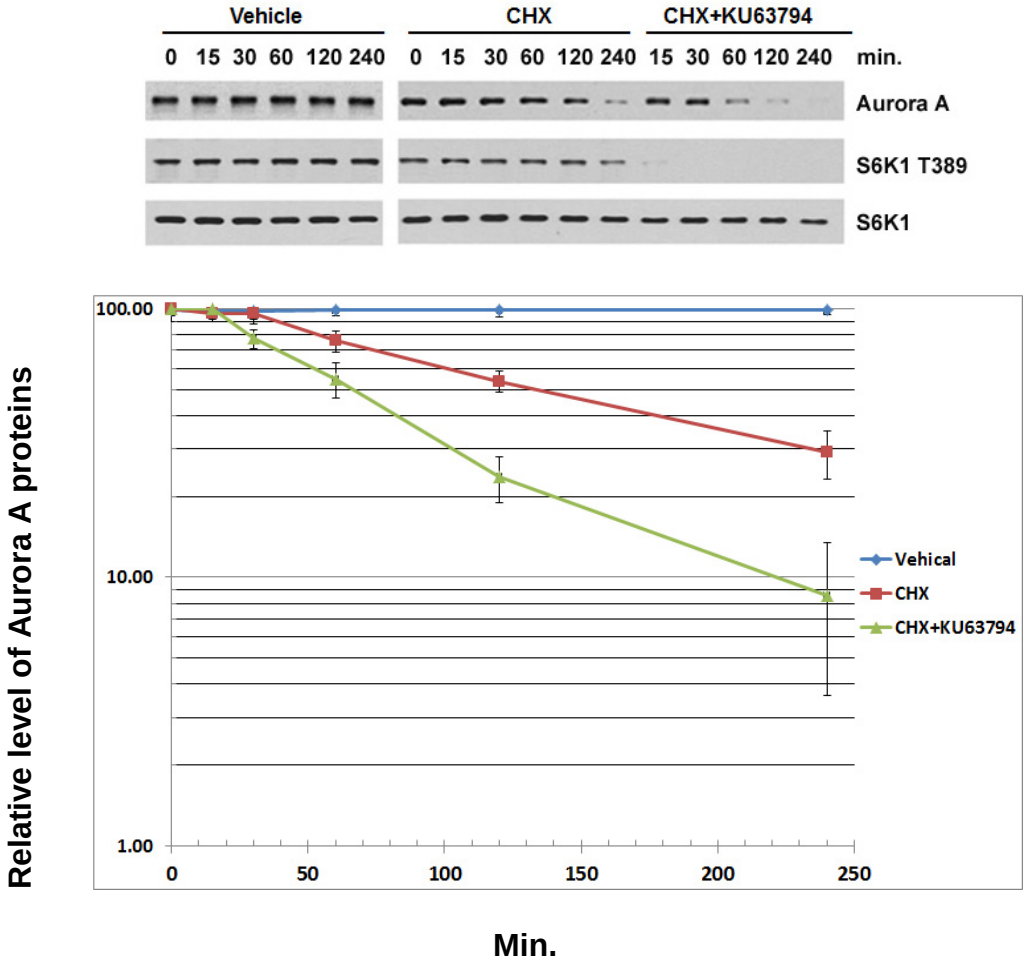

Figure 4.11 Blocking mTOR accelerates Aurora A protein destruction.

A. HeLa cells were pre-treated with protein synthesis inhibitor CHX, 50 μg/ml for 30 minutes,before being exposed to mTOR inhibitor KU63794 1 μM or vehicle DMSO up to 4 hours. Cell lysates were harvested at indicated time points after KU63794 treatment (t=0) and equal amounts of the proteins were then subjected to SDS-PAGE, and Aurora A, S6K1 protein and its T389 phosphorylation were measured by immunoblotting assays.

B. Diagram showing quantitation of Aurora A proteins from three independent experiments in a semi-log plot.

4.12 Inhibition of proteasome activity prevents mTOR-mediated Aurora A destruction

It is documented that APC induces Aurora A protein turn over, by promoting ubiquitination and subsequently degradation by the 26S proteasome in later M and G1 phases of the cell cycle [99]. This process is regulated by mTOR signaling remains to be ascertained. To address this issue, I used a well-known proteasome inhibitor MG132 [156-158], to block protein degradation and used KU63794 to block mTOR signaling to see if mTOR affects proteasome-mediated Aurora A degradation. The cells were pre-treated with MG132 for 30 minutes then exposed to mTOR inhibitor KU63794. Three hours post MG132 treatment (two and half hours post KU63794) lysates were subject to immunoblotting assays (Figure 4.12). The results showed that (1) inhibiting proteasome activity accumulated Aurora A proteins as previously reported [99]. (2) Suppression of mTOR activity decreased level of the Aurora A, consistent with my previous data. (3) Pre-treated with MG132 prevented Aurora A destruction from KU63794 treatment, shown in Figure 4.10, indicating mTOR regulates Aurora A protein stability via a proteasome-mediated pathway.

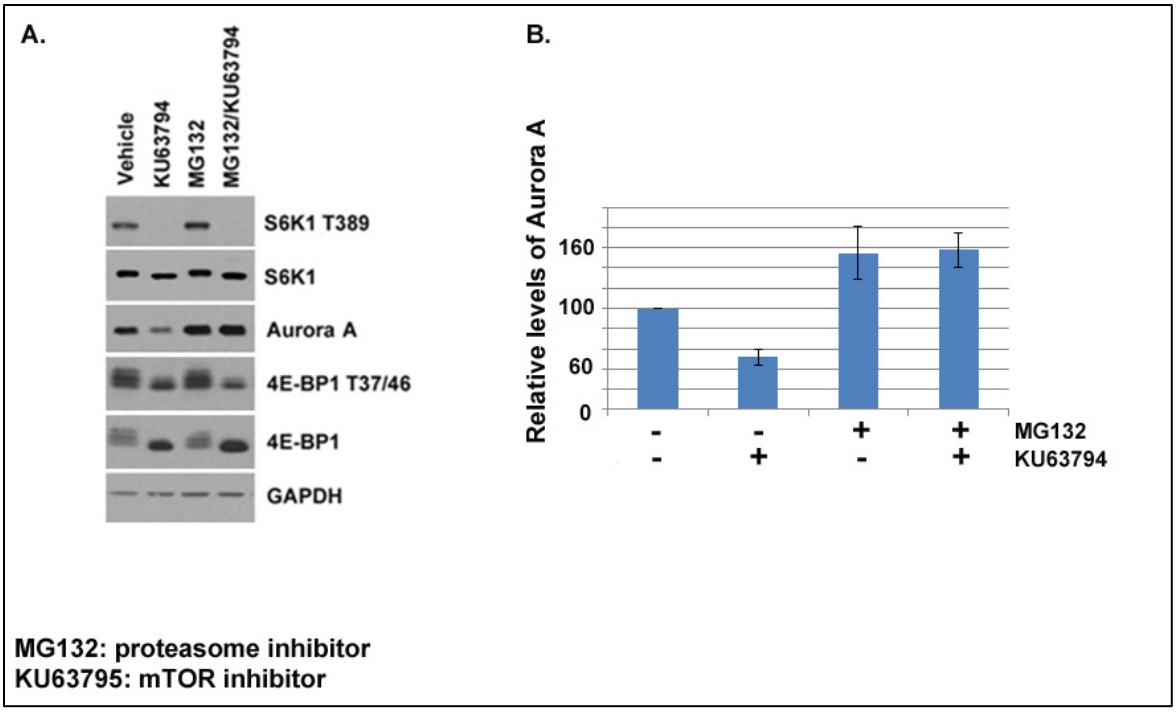

Figure 4.12 Inhibition of proteasome activity prevents mTOR-mediated Aurora A destruction.

A. HeLa cells were pre-treated with proteasome inhibitor MG132 3 μM for 30 minutes before being exposed to mTOR inhibitor KU63794 or vehicle DMSO for additional 2.5 hours. Cell lysates were harvested and equal amounts of the proteins were then subjected to SDS-PAGE, and Aurora A, S6K1, 4E-BP1, and their phosphorylation were measured by immunoblotting using specific antibodies.

B. The relative levels of Aurora A proteins from three independent experiments are shown in the histogram.

4.13 mTOR-mediated destruction of Aurora A is not completely dependent on APC activator Cdh1

It is well-known that Cdh1, also called homolog of Drosophila fizzy-related gene 1 or FZR1, functions as an activator of APC/C at later mitosis (anaphase) and subsequent G1 to destroy Aurora A protein [111] [159]. To examine the mechanism of mTOR action, I asked whether Cdh1 is involved in mTOR mediated Aurora A destruction. To answer this question, I first examined Cdh1 specificity and efficiency of shRNAs. From five shRNAs which target different coding regions of Cdh1 genes, I identified that three shRNAs effectively knocked down Cdh1 expression (Figure 4.13.1). By blast search, two shRNAs, #3 and #4, have no or only minimal off-target binding. Thus, these two shRNAs were selected to use in the following studies.

To look directly at the mechanism of APC/cdh1-dependent, mTOR-mediated Aurora A degradation, I knocked down Cdh1 by transducing lenti-viral Cdh1 shRNAs to the cells for 60 hours then treating with or without mTOR inhibitor KU36794 for additional 12 hours. The immunoblotting showed in Figure 4.13.2 that Aurora A accumulated in the Cdh1 knocked down sample and Aurora A level was diminished in the mTOR suppressed cells, as expected. However, knocking down Cdh1 did not completely prevent Aurora A degradation from mTOR inhibition. Considering Cdh1 only interacts with the D-box of Aurora A, I reasoned that additional Aurora A sequence(s) or signaling pathway(s) may be involved in the protein destruction.

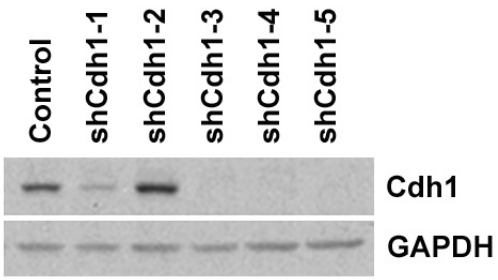

Figure 4.13.1 Efficiency of Cdh1 shRNAs

Five individual lenti-viral Cdh1 shRNAs, which target different coding regions of the Cdh1gene, were generated, see Material and Method. HeLa cells were transduced with 0.5 ml of individual lenti-viruses that contain specific shRNAs. At 72 hours post transduction, the cellular extracts were harvested and subject to immunoblotting analysis using antibodies that specifically recognize Cdh1 protein. GAPDH was used as a loading control.

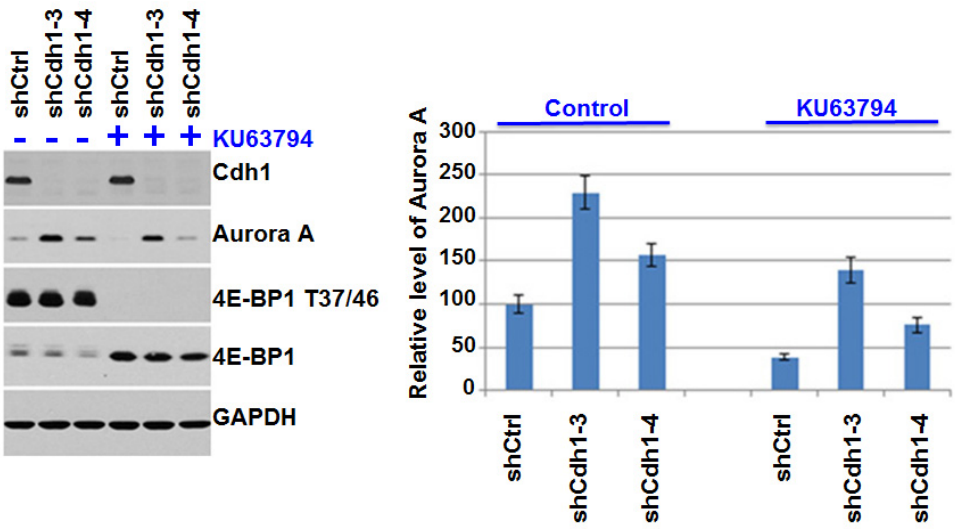

Figure 4.13.2 mTOR-mediated degradation of Aurora A is not completely dependent on APC activator Cdh1.

A. Knockdown Cdh1 was conducted by transduction of lenti-viral shRNAs indicated above in HeLa cells. At 60 hours post transduction, the cells were treated with mTOR inhibitor KU36794 1μM or vehicle DMSO for 12 hours. Cell lysates were harvested and equal amounts of the proteins were then subjected to SDS-PAGE, Cdh1, Aurora A, S6K1, 4E-BP1, and 4E-BP1 T37/46 were measured by immunoblotting using specific antibodies.

B. The data from three independent experiments was pooled and shown as mean ± standard deviation.

4.14 Suppression of PP2A phosphatase rescues mTOR-mediated Aurora A destruction

Protein phosphatase 2A (PP2A) is a key enzyme for metaphase-anaphase transition in mammalian cells [160-164]. PP2A was found to co-localize with and interacted with Aurora A in the centrosomes [165, 166] during interphase [167, 168] as well as mitosis [169] and is responsible for Aurora A serine 51 (S51) phosphorylation status [75]. Therefore I hypothesized that mTOR regulates Aurora A degradation via PP2A. To test this possibility, I used a serine/threonine phosphatase inhibitor okadaic acid (OA), which at low concentration preferentially inhibits the PP2A activity [164, 170]. In this experiment, HeLa cells were pre-treated with OA 1 nM for 15 minutes prior to exposure to KU63794 for an additional hour. The immunoblotting showed at Figure 4.14 that inhibition of mTOR activity reduced the amount of Aurora A, compared to the vehicle control, consistent with my previous data. Importantly, treating cells with OA prior to addition of KU63794 rescued Aurora A proteins from degradation, supporting my hypothesis that action of mTOR in stabilizing Aurora A protein results from inhibition of PP2A phosphatase activity.

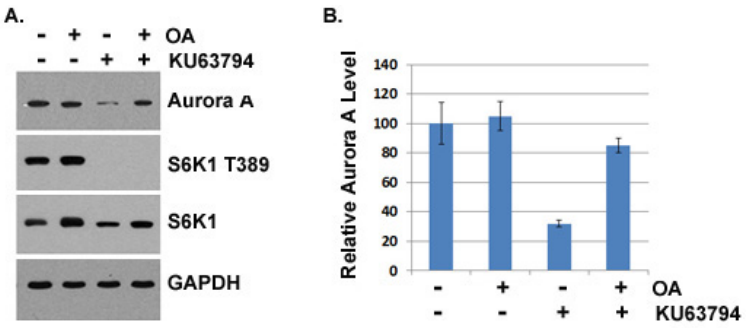

Figure 4.14 Inhibition of phosphatase PP2A rescues mTOR-mediated Aurora A destruction.

A. HeLa cells were pre-treated with PP2A inhibitor OA 1 nM for 15 minutes prior to exposure to mTOR inhibitor KU63794 1μM or vehicle DMSO for 1 hour. Cell lysates were harvested and equal amounts of the proteins were then subjected to SDS-PAGE, Aurora A, S6K1 and its residue T389 phosphorylation were measured by immunoblotting assays using specific antibodies.

B. Graph of Aurora A protein levels from three experiments.

4.15 PP2A substrate Aurora-A Ser51 is responsive to mTOR-mediated protein destruction

It was reported that in Xenopus either substitution of S53 (corresponding to human S51) phosphorylation at N- terminal activation box (A-box) by a residue mimicking phosphorylation or a single point mutation in the degradation box (D-box) is sufficient to stabilize Xenopus Aurora A. The former mutation presumably prevents the unmasking of the D-box by dephosphorylation of the A-box [92]. It appears to take place in mammalian cells in vivo. Horn, et al. showed that PP2A dephosphorylates S51 in the A-box, leading to Aurora A degradation [119]. My data described above support the idea that mTOR inhibits PP2A activity to stabilize Aurora A protein. Thus, I reasoned that mTOR might affect the

phosphorylation status of Aurora A S51 and influence the protein stability. To test this possibility, I first transiently transfected constructs expressing either wild type (WT) Aurora A or one of two mutants, Aurora A S51A (non-phosphorylated) and Aurora A S51D (phosphomimic), and compared their sensitivities to degradation under mTOR inhibition. Consistent with the results reported previously, Aurora A S51A mutant expression was lower than that of Aurora A S51D or WT, as judged by the immunoblotting (Figure 4.15). This may be because the S51A mutant is unable to protect the D-box of the kinase from APC/C proteasome-mediated degradation.

Next, I examined whether mTOR controls Aurora A destruction via phosphorylation of Aurora A S51. HeLa cells described above (transfected with AURKA mutants for 24 hours) were treated with or without mTOR inhibitor KU63794 for an additional 2 hours. Interestingly, inhibition of mTOR accelerated Aurora A degradation in the S51A mutant cells, but not in the S51D (Figure 4.15). This indicates that mTOR elevates phosphorylation level of S51 in the “activation-box” of Aurora A, which dictates Aurora A sensitivity to proteasomal degradation.

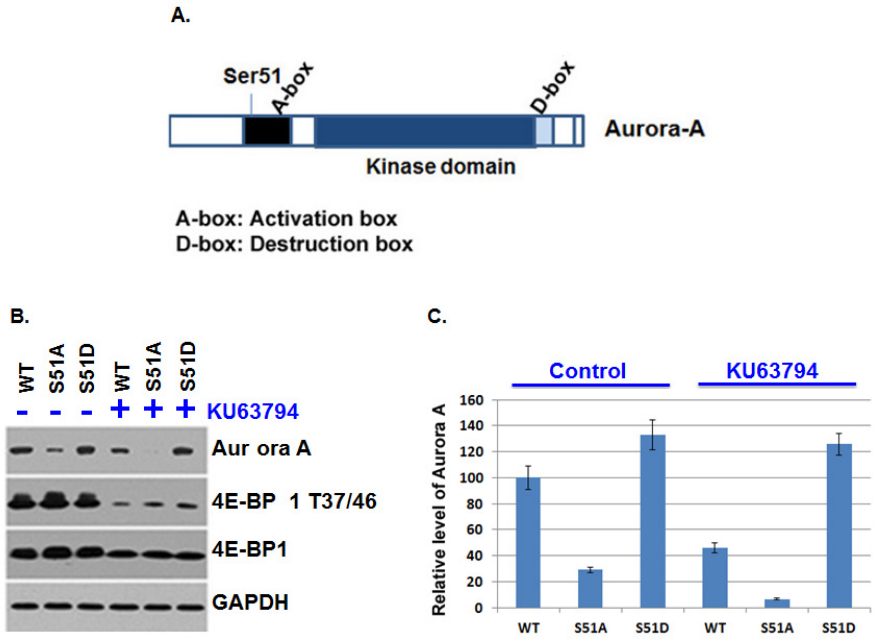

Figure 4.15 PP2A substrate Aurora A Ser51 is responsive to mTOR-mediated protein destruction

A.Domains of Aurora A protein

B.HeLa cells were transiently transfected with Aurora A WT or unphosphorylatable S51A or phosphomimetic S51D mutant for 24 hours, then treated with or without mTOR inhibitor KU63794 for 2 hours. The cellular lysates were subjected to SDS-PAGE, and the protein levels of exogenous Aurora A (Flag-Aurora A) and endogenous 4E-BP1 and GAPDH were determined by immunoblotting assays.

C. Aggregate data from three independent experiments is shown.

4.16 Summary

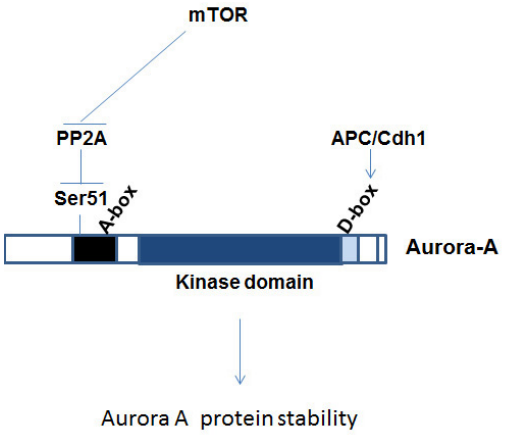

Figure 4.16 Model of mTOR/PP2A/Aurora A Ser15 axis for Aurora A protein stability

mTOR controls Aurora A destruction by inactivating PP2A, elevating the phosphorylation level of Ser51 in the “activation-box” of Aurora A, preventing the unmasked D box from APC/C Cdh1-mediated proteasome degradation. Thus, mTOR enhances stability of Aurora A protein.

In this study, I demonstrated that suppressing mTOR activity impacted the G2-M transition and reduced levels of M-phase kinases, Aurora A. mTOR inhibitors did not affect Aurora-A mRNA levels. However, translational reporter constructs showed that mRNA containing a short, simple 5’-untranslated region, rather than a complex structure, is more responsive to mTOR inhibition. mTOR inhibitors decreased Aurora A protein amount whereas blocking proteasomal degradation rescues this phenomenon, revealing that mTOR affects Aurora A protein stability. Inhibition of protein phosphatase, PP2A, a known mTOR substrate and Aurora A partner, restored mTOR-mediated Aurora A abundance. Finally, a non-phosphorylatable Aurora A mutant was more sensitive to destruction in the presence of mTOR inhibitor. These data strongly support the

notion that mTOR controls Aurora A destruction by inactivating PP2A and elevating the phosphorylation level of Ser51 in the “activation-box” of Aurora A, which dictates its sensitivity to proteasomal degradation.

4.17 Discussion

mTOR is a master regulator of cell metabolism, growth, proliferation and survival [9, 39, 171]. The mechanisms of mTOR actions are mainly through transcription, translation and protein stability. While these mTOR functions have been well-documented in the regulation of the G1 phase of the cell cycle, whether/how mTOR regulates the G2/M phases are just beginning to be understood.

During my early studies, I demonstrated that mTOR-induced phosphorylation of S6K1 and 4E-BP1 starts to increase in S phase and peaks at G2/M. During the course of my research, other investigators also observed that mTOR downstream kinase S6K1 and its substrate S6 were highly phosphorylated during lymphoma cell mitosis [82, 172]. The immunohistochemistry staining in cancer cells revealed that Serine 2481 autophosphorylation of mTOR couples with chromosome condensation and segregation during mitosis in breast cancer core biopsies [173]. TSC2 maintains nuclear envelope structure and controls cell division [174]. All these findings supported my hypothesis that mTOR may regulates mitotic progression.

Using mTOR inhibitors as a tool, I demonstrated that mTOR impacts mitotic progression by decreasing mitotic marker phosphor-Histone H3 and reducing 4N chromosomal population in mitosis. This is consistent with a recent work by Ramirez-Valle and his colleagues that showed mitotic raptor promotes G2/M transition; overexpression of a raptor phosphorylation mutant caused G2 accumulation in HaCaT cells [175, 176].

However, which mitotic kinase mediated mTOR action and how it is regulated by mTOR remains to be uncovered. In my pilot study, immunoblotting showed Aurora A is most obviously decreased, among several mitotic kinases, following inhibition of mTOR. Considering its critical roles in mitosis progression and tumorigenesis [120, 177-179], Aurora A was selected as a target for mTOR regulation in my study.

4.17.1 mTOR does not affect Aurora A transcription.

One of the known mechanisms for Aurora A overexpression in cancers is through RNA transcriptional up-regulation [94, 180]. Since mTOR was reported to regulate transcription [20], I examined mTOR effects on AURKA mRNA levels. Quantitative PCR showed that AURKA mRNA levels were not alternated by mTOR inhibitor rapamycin. Consistently, the transcripts also remain unchanged in the cells treated with three additional ATP-competitive mTOR kinase inhibitors. These data strongly suggest that mTOR does not affect Aurora A transcription.

4.17.2 Does a short and simple or long and complex, mRNA structure preferentially regulated by mTOR?

Genomic analysis revealed that 35-94% of cancers undergo alterative splicing, and lots of cancer-specific transcript variants were identified [181, 182]. Chien-Hsien Lai, et al. reported that EGF mediated translational regulation of AURKA contains non-coding exons I and II within the 5' UTR [95]. As shown in Figure 1.6, AURKA 5'UTRs that contain exon II are longer and possess an upstream ORF that is out of frame with the main initiating ATG. Thus it appeared that a long, complex mRNA with a secondary structure might be sensitive to mTOR regulation.

Recently, Thoreen et al. (2012) performed transcriptome-wide profiling in mouse embryonic fibroblasts cells to determine the effect of mTOR inhibition on mRNA translation (ribosome loading) [69]. They found no evidence for a previously postulated theory that mTORC1 regulates the translation of mRNAs with highly structured and complex 5' untranslated regions. These controversial results raised a question whether short or longer mRNAs are sensitive to mTORC1 regulation. In ours hands, the luciferase assays consistently showed that a short and simple (splice variant 6), rather than long and complex (splice variant 5), 5'-UTR of AURKA is sensitive to mTOR signaling, supportive of the study by Thoreen and colleagues.

Since the variant 6 is sensitive to mTOR manipulation, it would be interesting to identify specific types of cancers which predominantly express this

or other short versions of AURKA UTR. This information may provide strategy chemotherapy against certain tumors.

As the variant 6 is a minor expressed form of Aurora A mRNA in HeLa cells, additional pathway(s) must be required for mTOR regulation of Aurora A as total Aurora A proteins were dramatically reduced by mTOR inhibition.

4.17.3 How does mTOR affect Aurora A protein stability?

It is well known that Aurora A protein degradation is through APC/C-mediated ubiquitination-proteasome pathway. I therefore questioned whether mTOR mediated Aurora A stability via this pathway. Using protein synthesis inhibitor CHX and proteasome inhibitor MG132, I found that suppression of proteasome activity prevents mTOR-mediated Aurora A destruction, implying that mTOR is involved in the regulation of Aurora A protein stability. Since Aurora A is reported to be ubiquitinated by APC in order to be recognized by the 26S proteasome system for destruction, it would be interesting to examine the ubiquitination status of Aurora A under mTOR inhibition to further confirm this finding. An experimental design for this purpose will be discussed in the “Future Direction” section.

4.17.4 How does PP2A involve in mTOR - mediated Aurora A protein stability ?

Firstly, does mTOR may regulate Aurora A degradation via PP2A or it might just be that PP2A regulates an essential step in Aurora A degradation, independent of mTOR? To answer this question, exogenous S51 mutants were expressed in HeLa cells that were pre-treated with PP2A inhibitor, OA prior to exposing cells to mTOR inhibitor. However, a technical challenge was that these, exogenous S51A or S51D mutants require a 18-24 hours period time post transfection, to become expressed while OA can only be administered to cells for a short time, less than 2 hours.

It is known that mTOR inhibits PP2A activity while treatment of cells with rapamycin stimulated PP2A activity [183]. The mechanism of mTOR action to PP2A is believed through Tap42p in Saccharomyces cerevisiae (TAP42) and alpha4 in mammalian cells). In yeast, Tor regulates PP2A by phosphorylating TAP42, causing a redistribution of the PP2A catalytic subunit into a complex with TAP42 and away from the normal A and B regulatory subunits [184]. The binding of PP2A toTAP42 leads to the inhibition of PP2A activity or a dramatic change in its substrate specificity [185]. In mammalian cells, the pool of PP2A was found associated with alpha 4. Although a small portion of the cellular pool of PP2A is present in a complex with alpha4, the latter plays a crucial role to affect PP2A activity [186]. Using PP2A inhibitor OA, I demonstrated that PP2A inhibition impaired the mTOR-dependent Aurora A destruction, indicating that mTOR

mediated Aurora A stability via a dephosphorylation pathway and that PP2A may be involved in this event.

Viallet et al. reported that PP2A and Aurora A proteins are co-localized at the centrosome during mitosis and following G1 phase [119]. PP2A directly dephosphorylates Aurora A Ser51 and triggers Aurora A degradation [119] whereas reducing PP2A enzymatic activity with OA stabilizes Aurora A protein. In the current study, based on the finding that inhibition of mTOR accelerates degradation of non-phosphorylatable Aurora A S51A mutant, I found that mTOR prevent Aurora A S51 dephosphorylation, This conclusion is further supported by the observation that the phosphor-mimic S51D mutant restored the Aurora A protein levels.

By 2007, studies had shown that in Xenopus either substitution of S53 (corresponding to human Aurora A S51) of the A box by a residue mimicking phosphorylation or a single point mutation in the degradation box is sufficient to stabilize Xenopus and mammalian Aurora A [109, 119]. The former mutation presumably prevents the unmasking of the D-box by dephosphorylation of the A-box. As the D-box is involved in recognition by the APC/C Cdh1 complex, its alteration also prevents degradation. My data demonstrated that mTOR is an upstream regulator of Aurora A, and suggest that mTOR controls Aurora A S51 phosphorylation, perhaps indirectly, and consequently modulates Aurora A protein stability. While mTOR regulated Aurora A dephosphorylation, the kinase that catalyzes Aurora A S51 phosphorylation is unknown. It would also be

interesting in future to investigate the role of Aurora A S51 in the cell cycle control.

Recent studies by others have shown that protein phosphatase PP6 also interacts with alpha4 and mimics PP2A activity during mitosis [187-191]. PP6 acts as an Aurora A T-loop phosphatase against known Aurora A activator targeting protein for Xenopus kinesin-like protein 2 (TPX2), a key protein required for targeting Aurora A to the mitotic spindle [187, 192]. Depletion of PP6 catalytic subunit with RNAi led to abnormal chromosome segregation in anaphase, which was favorable to stabilize Aurora A interacting with its activator TPX2 [187]. The latter not only stimulates autophosphorylation at T loop residue Thr288, autoactivation of the enzyme [193], and targets Aurora A to the spindle, but also affects Aurora A protein stability [194]. These studies suggest that PP6 affects activity and stability of Aurora A.

More interestingly, mTOR seems to be associated with PP6. In yeast, and probably in higher eukaryotes, mTOR signals through Tap42p/alpha 4 to regulate protein phosphatases. Phosphorylating Tap42p/alpha 4, mTOR abrogates dephosphorylation of the downstream effectors by PP2A and/or PP6, resulting in their increased phosphorylation and activity [195]. Thus, it would be interesting to examine whether PP6 is also involved in mTOR-mediated Aurora A protein activity and stability. This is further discussed in the “future direction” section.

4.17.5 Why would mTOR stabilize Aurora A during M phase?

mTOR stabilization of Aurora A perhaps helps maintain the mitotic spindle checkpoint during the cell cycle. This is supported by Astrinidis' study [196] on how TSC2 loss (equivalent to mTOR activation) leads to regulation of mitosis and might be tightly regulated by feedback from M phase kinases PLK1. It was shown that Hamartin (TSC1 product) negatively regulates the protein levels of PLK1 in TSC1/2 mouse embryonic fibroblasts and increases the number of centrosomes. Thus TSC1/2 and mTOR signaling may regulate mitosis checkpoint during the cell cycle.

In summary, this study showed that mTOR activity remains high at G2/M phase of the cell cycle. Inhibition of mTOR impacted mitosis progression. This study demonstrated that mTOR regulates Aurora A mitotic protein kinase expression and protein stability. Depletion of mTOR activity did not affect Aurora-A message RNA levels. Suppressing mTOR modestly inhibited Aurora A mRNA translation, particularly the less-abundant short 5'UTR variant 6. mTOR controls Aurora A stability by inactivating PP2A and elevating the phosphorylation level of Ser51 in the "activation-box" of Aurora A, which dictates its sensitivity to proteasomal degradation. This is the first report demonstrating that mTOR signaling regulates Aurora A protein expression and stability and provides a better understanding of how mTOR regulates mitotic kinase expression and coordinates cell cycle progression.

FUTURE DIRECTIONS

It was previously reported that PP6 interacts with Aurora A at the centrosome and might affect both the activity and stability of Aurora A [187]. Since mTOR may additionally influence PP6 activity [195], it would be interesting to examine whether PP6 is involved in mTOR-mediated Aurora A destruction. Technically, it is suitable to carry out further study using our current model system since all the PP6 subunits exist in HeLa cells [187]. Use of RNAi approach to knock down PP6 mRNAs, in the presence or absence of mTOR inhibitor, would help us to answer this question.

Since Aurora A splice variant 6 is sensitive to mTOR inhibition, then the translation of Aurora A in tumor cells expressing each variant with a short 5'UTR that lacks uORFs (variants 2, 4, 6) may be suppressed by mTOR inhibitors. It would be interesting to test this hypothesis as it may identify a means of screening tumors for sensitivity to treatment with mTOR kinase inhibitors.

APPENDIX PCR PRIMERS

Primer	Sequence
Aurka1rev	5'-TGGAGTGAGACCCTCTAGCTGTA
Aurka2rev	5'-CCATGATGCCTCTAGCTGTAAT
Aurka3rev	5'-TGGAGTGAGACCCCGTCGGCTC
Aurka4rev	5'-CCATGATGCCCGTCGGCTCCCA
Aurka5rev	5'-TGGAGTGAGACCCTGCGACCCA
Aurka6rev	5'-GATCGGTCCATGATGCCTGCGA
Aurka1fwd	5'-GTCAACCAATCACAAGGCAGC

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PUBLICATIONS

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MEETING ABSTRACTS

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